PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/505, A61K 38/18	A2	(11) International Publication Number: WO 96/40772
		(43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/US96/09469

(22) International Filing Date: 6 June 1996 (06.06.96)

(30) Priority Data:
08/484,135 7 June 1995 (07.06.95) US

(71) Applicant *(for all designated States except US)*: JOHNSON & JOHNSON [US/US]; One Johnson & Johnson Plaza, New Brunswick, NJ 08933-0001 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: JOHNSON, Dana, L. [US/US]; 1343 Lonely Cottage Road, Upper Black Eddy, PA 18972 (US). ZIVIN, Robert, A. [US/US]; 6 Glenbrook Court, Lawrenceville, NJ 08648 (US).

(74) Agents: CIAMPORCERO, Audley, A., Jr. et al.; Johnson & Johnson, One Johnson & Johnson Plaza, New Brunswick, NJ 08933-0001 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: AGONIST PEPTIDE DIMERS

(57) Abstract

The present invention is directed to the dimerization of agonists and antagonists of cell surface receptors and particularly to peptide dimers which behave as cell surface receptor agonists in their dimeric form. Such receptors belong to the dimerization-mediated activation class often observed among receptors for growth and differentiation factors. The agonists of this class of receptors is understood to effect dimerization of the receptor and thus signal initiation. The present invention exemplifies dimers of erythropoietin (EPO) agonists and antagonists comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid or Hoc; X_4 can be R, H, L or W; X_5 can be M, F, or I; X_6 is idependently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid or Hoc, provided that either X_3 or X_8 is C or Hoc.

AGONIST PEPTIDE DIMERS

FIELD OF THE INVENTION

The present invention is directed to the dimerization of agonists and antagonists of cell surface receptors and particularly to peptide dimers which behave as cell surface receptor agonists in their dimeric form. Such receptors belong to the dimerization-mediated activation class often observed among receptors for growth and differentiation factors. The agonists of this class of receptors is understood to effect dimerization of the receptor and thus signal initiation. The present invention exemplifies dimers of erythropoietin (EPO) agonists and antagonists comprising a core amino acid sequence of $X_3X_4X_5GPX_8TWX_7X_8$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid or Hoc; X_4 can be R, H, L or W; X_5 can be M, F, or I; X_8 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid or Hoc, provided that either X_3 or X_8 is C or Hoc.

BACKGROUND OF THE INVENTION

Erythropoietin (EPO) is a glycoprotein hormone with an approximate molecular weight of 34,000 daltons. The primary role of EPO, which is synthesized in the kidneys of mammals, is to stimulate mitotic cell division and differentiation of erythrocyte precursor cells. As a result, EPO acts to stimulate and to

Claus-Walker et al. (1984) Arch. Phys. Med. Rehabil. 65:370-374); space flight (See, Dunn et al. (1984) Eur. J. Appl. Physiol. 52:178-182); acute blood loss (See, Miller et al. (1982) Brit. J. Haematol. 52:545-590); aging (See, Udupa et al. (1984) J. Lab. Clin. Med. 103:574-588); various neoplastic disease states accompanied by abnormal erythropoiesis (See, Dainiak et al. (1983) Cancer 5:1101-1106); and renal insufficiency (See, Eschbach et al. (1987) N. Eng. J. Med. 316:73-78).

Although purified, homogenous EPO has been characterized, little is known about the mechanism of EPO-induced erythroblast proliferation and differentiation. The specific interaction of EPO with progenitor cells of immature red blood cells, platelets, and megakaryocytes has not been described. This is due in part, to the small number of surface EPO receptor molecules on normal erythroblasts and on the erythroleukemia cell lines. See Krantz and Goldwasser (1984) Proc. Natl. Acad. Sci. USA, 81:7574-7578; Branch et al. (1987) Blood 69:1782-1785; Mayeux et al. (1987) FEBS Letters 211:229-223; Mufson and Gesner (1987) Blood 69:1485-1490; Sakaguchi et al. (1987) Biochem. Biophys. Res. Commun. 146:7-12; Sawyer et al. (1987) Proc. Natl. Acad. Sci. USA 84:3690-3694; Sawyer et al. (1987) J. Biol. Chem. 262:5554-5562; and Todokoro et al. (1988) Proc. Natl. Acad. Sci. USA 84:4126-4130. The DNA sequences and encoded peptide sequences for murine and human EPO receptor proteins have been described. See, D'Andrea et al. PCT Patent Publication No. WO 90/08822 (published 1990).

dimers have two 'monomeric' peptide units of 10 to 40 or more amino acids, preferably 14 to about 20 residues in length, comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) where each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_6 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc. Preferably, the monomeric peptide unit of the dimer comprises a core sequence $YX_2X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 2) where each amino acid is indicated by standard one letter abbreviation; each X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

More preferably, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3), where each amino acid is indicated by standard one letter abbreviation; each X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is

SCHFGPLTWVCK (SEQ ID NO: 18).

Other particularly preferred monomeric peptide units of the present dimers include peptides comprising a core sequence of the formula $(AX_2)_nX_3X_4X_5GPX_8TWX_7X_8$ (SEQ ID NO: 19) wherein X_2 through X_8 are as previously defined herein (SEQ ID NO: 2), n is 1 or 0 and A is any one of the naturally occurring L-amino acids except Y (tyrosine); n is defined herein as the number of occurrences of (AX_2) which can be 1 or none in the core sequence. When (AX_2) is present, i.e. when n = 1, A is not tyrosine and A is not any non-naturally occurring aromatic amino acid analog. Such monomeric peptide units of the dimers of this invention can be prepared by truncating the peptides of Fig. 9, for example, from the N-terminus to delete the Y, tyrosine residue in SEQ ID NOS. 21 - 93. Such monomeric peptides can also be prepared by substitution of Y in position A in the peptides of Fig. 9.

In accordance with the present invention the monomeric units of the dimers can be the same or different.

In a preferred embodiment polyethylene glycol (PEG) is employed as a linker to form the dimeric peptides of the present invention through a covalent bond.

In another embodiment, the present invention is directed to pharmaceutical compositions comprising at least one dimer peptide of the invention and a pharmaceutical carrier.

Fig. 2 shows a major peak, with a retention time of 48 minutes, following purification of the dimerized EPO peptide, (SEQ ID NO: 8).

Fig. 3 depicts the MALDI-TOF mass spectral analysis of the dimerized peptides, including peptide (SEQ ID NO: 8), GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13) and SCHFGPLTWVCK (SEQ ID NO: 18).

Fig. 4 shows the SDS-PAGE analysis of DPDPB crosslinking of EPO binding protein (EBP) in the presence and absence of EPO agonist peptides.

Fig. 5 demonstrates equilibrium EPO binding to immobilized EPO binding protein. Panel A represents the equilibrium binding data and Panel B (inset) is the linear transformation (Scatchard) of the data set in Panel A.

Fig. 6 depicts the results of a competitive binding assay run on the EPO agonist peptide (SEQ ID NO: 8) in competitive binding with [^{125}I]EPO to EBP beads (Panel A); and EPO responsive cell proliferation studies in FDC-P1 derived cell lines containing either a human (Panel B) or murine EPO receptor (Panel C).

Fig. 7 is a graphic representation of the results of the exhypoxic mouse bioassay; stimulation of the incorporation of [^{59}Fe] into nascent red blood cells by EPO, peptide (SEQ ID NO: 8) (Panel A) and peptide (SEQ ID NO: 8) dimer (Panel B).

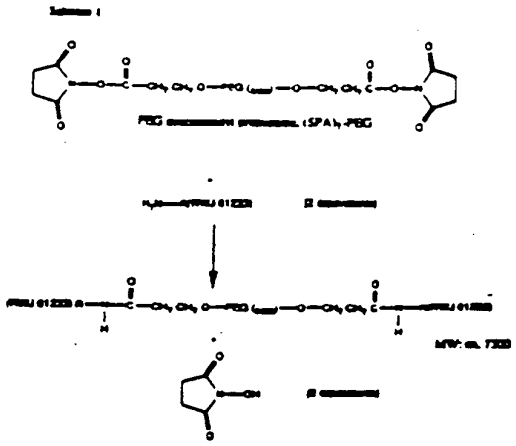
Fig. 8 demonstrates the effect of PEG dimerization of peptide (SEQ ID NO: 18) activity in EPO responsive cell proliferation studies in FDC-P1 derived cell lines containing a human EPO receptor.

cell-surface receptors in vitro and in vivo. Such receptors include, for example, EPO, GM-CSF, G-CSF, M-CSF, GH, EGF, PDGF, VEGF, Insulin and FGF. Other receptors which are activated by heterodimerization or multimerization may also be subject to activation by this mechanism including, IL-3, IL-5, IL-6, IL-2 and TPO. The dimers of the present invention have two 'monomeric' peptide units of 10 to 40 or more amino acids, preferably 14 to about 20 amino acid residues in length. In a preferred embodiment, these monomeric peptide units comprise a core sequence of amino acids $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) where each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_6 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc. Preferably, the monomeric peptide unit of the dimer comprises a core sequence $YX_2X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 2) where each amino acid is indicated by standard one letter abbreviation; each X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

GGDYHCRMGPLTWVCKPLGG (SEQ ID NO: 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID NO: 10);
GGVYACRMGPITWVCSPLGG (SEQ ID NO: 11);
VGNYMAHMGPITWVCRPGG (SEQ ID NO: 12);
GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13);
GGLYACHMGPMTWVCQPLRG (SEQ ID NO: 14);
TIAQYICYMGPETWECRPSPKA (SEQ ID NO: 15);
YSCHFGPLTWVCK (SEQ ID NO: 16);
YCHFGPLTWVC (SEQ ID NO: 17); and
SCHFGPLTWVCK (SEQ ID NO: 18).

The dimer peptides of the present invention exhibit increased biological potency in vitro and in vivo relative to the monomeric agonists from which the dimers are derived. Moreover, cell surface receptor antagonists can be 'converted' to cell surface receptor agonists in accordance with the present invention. Specifically, a cell surface receptor antagonist can be dimerized with PEG or another appropriate linker which permits mutual binding of the monomeric moieties with the receptors. As a result, the dimer exhibits effective binding to the target receptor and behaves as an agonist. Accordingly, the dimers of this invention demonstrate enhanced biological potency in vitro and in vivo relative to their monomeric forms.

The dimer peptides of the present invention bind to and biologically activate the cell surface receptor or otherwise behave as agonists and are preferably formed by employing polyethylene glycol as a linker between the monomeric peptide units described herein. While other conventional chemical systems can



Dimerization and especially pegylation in a head-to-head (amino to amino terminus) or head-to-tail (amino to carboxyl terminus) configuration is preferred relative to internal covalent binding of the monomeric peptides. The 'monomer' units of the dimer peptides of the present invention can be the same or different, although the same are preferred.

The monomeric peptides which are used to form the dimers of the present invention can be prepared by classical chemical methods well known in the art. The standard methods include, for example, exclusive solid phase synthesis and recombinant DNA technology. See, e.g. Merrifield (1963) J. Am. Chem. Soc. 85:2149. Solid phase synthesis is typically commenced from the C-terminal end of the peptide using an α-amino protected resin. A suitable starting material can be prepared by attaching the required α-amino acid to a chloromethylated resin (such as BIO-BEADS SX-1, Bio Rad Laboratories, Richmond, CA), a hydroxymethyl resin, (described by Bodonszky et al. (1966) Chem. Ind.

Boc. The side chain protecting groups for Lys include Cbz, 2-chlorobenzyloxycarbonyl (2-Cl-Cbz), 2-bromobenzyloxycarbonyl (2-BrCbz), Tos, or Boc.

After removal of the α-amino protecting group, the remaining protected amino acids are coupled stepwise in the desired order. Each protected amino acid is generally reacted in about a 3-fold excess using an appropriate carboxyl group activator such as 2-(1H-benxotriazol-1-yl)-1,1,3,3tetramethyluronium hexafluorophosphate (HBTU) or dicyclohexylcarbodiimide (DCC) in solution of methylene chloride (CH_2CI_2), or dimethyl formamide (DMF) mixtures.

After the desired amino acid sequence has been completed, the desired peptide is decoupled from the resin support by treating the mixture with a reagent such as trifluoroacetic acid (TFA) or hydrogen fluoride (HF). These reagents not only cleave the peptide from the resin, but also cleave all remaining side chain protecting groups. When the chloromethylated resin is used, hydrogen fluoride treatment results in the formation of the free peptide acids. When the benzhydrylamine resin is used, hydrogen fluoride treatment results directly in the free peptide amide. Alternatively, when the chloromethylated resin is employed, the side chain protected peptide can be decoupled by treatment of the peptide resin with ammonia to give the desired side chain protected amide or with an alkylamine to give a side chain protected alkylamide or dialkylamide. Side chain protection is then removed in the usual fashion by treatment with hydrogen fluoride to give the free amides, alkylamides, or dialkylamides.

flexibility leading to fewer barriers to effective receptor interaction and subsequently receptor activation. This is also indicated for molecules which can bind but not activate a receptor subtype in that such molecules can become more effective inhibitors of ligand binding.

The present invention further provides a method for altering a cell-surface receptor antagonist, a molecule exhibiting receptor binding but no biological activity, to behave as a cell-surface receptor agonist in vitro or in vivo. This method is achieved by dimerizing the antagonist molecule with an appropriate linker molecule such as PEG, other polymerized molecules or a peptide. In a preferred embodiment, an EPO antagonist, i.e. a peptide exhibiting receptor binding but no biological EPO activity can be altered by dimerization to obtain a dimer which behaves as an EPO receptor agonist. Thus, for example, in the case of EPO-R these include the monomeric peptide units of the present dimers comprising a core sequence of general formula $(AX_2)_nX_3X_4X_5GPX_8TWX_7X_8$ (SEQ ID NO: 19) wherein X_2 through X_8 are as previously defined herein, in (SEQ ID NO: 2), n is 1 or 0 and A is any one of the naturally occurring L-amino acids except Y (tyrosine); n is defined herein as the number of occurrences of (AX_2) which can be 1 or none in the core sequence. When X_2 is present, i.e., when n = 1, A is not tyrosine and A is not any non-naturally occurring aromatic amino acid analog. Such monomeric peptide units of the dimers of this invention can be prepared by truncating the peptides of Fig. 9, for example, from the N-terminus to

iodohydroxyphenylalanine, p-fluorohydroxyphenylalanine, p-amino-hydroxyphenylalanine act as EPO-R monomer agonists but substitution with threonine or alanine for tyrosine at position Y causes the monomer peptide to act as an EPO-R antagonist. However, when dimerized in accordance with the present invention, such dimers behave as EPO-R agonists. The monomeric peptide units identified at Fig. 9, for example, behave as EPO-R antagonists in the absence of tyrosine at position Y of the formula above. When such antagonists are dimerzied, the dimer behaves as an EPO-R agonist.

In a further embodiment of the present invention, pharmaceutical compositions comprising at least one of the dimers of this invention can be employed to therapeutically treat disorders resulting from deficiencies of biological factors such as EPO, GH, GM-CSF, G-CSF, EGF, PDGF, VEGF, insulin, FGF and the like. These pharmaceutical compositions may contain buffers, salts and other excipients to stabilize the composition or assist in the delivery of the dimerized molecules.

In a preferred embodiment, the present invention provides a method for treating disorders associated with a deficiency of EPO. The method is accomplished by administering at least one of the dimers identified herein for a time and under conditions sufficient to alleviate the symptoms of the disorder, i.e. sufficient to effect dimerization or biological activation of EPO receptors. In the case of EPO such methodology is useful in the treatment of end-stage renal failure/dialysis; anemia, especially associated

(nasal, vaginal, rectal, or sublingual) routes of administration in dosage forms appropriate for each route of administration.

Solid dosage forms for oral administration include capsules, tablets, pill, powders, and granules. In such solid dosage forms, the active compound is admixed with at least one inert pharmaceutically acceptable carrier such as sucrose, lactose, or starch. Such dosage forms can also comprise, as it normal practice, additional substances other than inert diluents, e.g., lubricating, agents such as magnesium stearate. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering, agents. Tablets and pills can additionally be prepared with enteric coatings.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, with the elixirs containing inert diluents commonly used in the art, such as water. Besides such inert diluents, compositions can also include adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring and perfuming agents.

Preparations according to this invention for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting,

EXAMPLE 1

SDS-PAGE gels (10-20% gradient SDS-PAG plates, 84 x 70 x 1.0 mm, Integrated Separation Systems, Natick, MA) were stained with Coomasie Brilliant Blue R-250 (BioRad). A commercial preparation of activated difunctional polyethylene glycol (PEG-succinimidyl-propionate, SPA2, MW ca. 3400) was purchased from Shearwater Polymers, Huntsville, AL as was the monofunctional reagent, methoxy-PEG-succinimidyl-proprionate, MW ca 5000. Peptide (SEQ ID NO: 8) and all other peptides were obtained from the Peptide Synthesis Facility RWJ-PRI, La Jolla, CA or Quality Controlled Biochemical, Hopkinton MA. These peptides were cyclized via oxidation of their intramolecular cysteines, amidated at the C-terminus and mass confirmed by FAB-MS. All were Ellman Reaction negative. Tris base was obtained from BioRad, Hercules, CA. (DPDPB) and trifluoracetic acid (HPLC grade) were obtained from Pierce Chemical Co., Rockford IL.

Mono-PEG conjugation of peptide GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8)

This example describes the preparation of mono-PEG conjugates of peptide (SEQ ID NO: 8), using the monofunctional amine reactive polymer analog m-SPA-PEG to be used as a control in experiments described herein. The reaction was carried out with polymer in excess (ca. 3 fold) by resuspending 142.5 mg (0.0286 mmol, MW ca. 5000) of polymer in 4 ml PBS at pH 7.5 and adding 20 mg peptide (SEQ ID NO: 8) (0.0095 mmol, MW 2092) dissolved

TABLE 1

Recovery Yield of Peptide Conjugation Reaction and Apparent Molecular Mass of Product

I.D. No.	Sequence	Mass	Conjugation Reagent	Main Product Mass (centroid m/z)	Yield (% of theoretical)
8	GGTYSCHFGPLTWVCKPQGG	2092	SPA2-PEG (MW ca. 3400)	7834	69
			m-SPA-PEG (MW ca. 5000)	7092 (peak 1)	-
				12036 (peak 2)	-
13	GGTYSCHFGPLTWVCKPQ	1978	SPA2-PEG	7560	54
20	Ac-GGTYSCHFGPLTWVCKPQGG	2133	SPA2-PEG	7862	30
14	GGLYACHMGPMTWVCQPLRG	2177	SPA2-PEG	7872	37
18	SCHFGPLTWVCK	1375	SPA2-PEG	6126	45

EXAMPLE 3

PEG dimerization of peptide GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8) (lot #2)

The modification of peptide (SEQ ID NO: 8) was carried out by resuspending 25 mg (0.0071 mmol) of polymer in 4 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 8) (0.0213 mmol, 45.8 mg, MW 2092) dissolved in 1 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 22 hours. At that time, the reaction was adjusted to a final concentration of 50 mM Tris by the addition of 1 M tris-HCl, pH 7.5. The reaction mixture was incubated on ice for 1 hour. The sample was subjected to analytical and preparative HPLC as described in Example 8. After preparative HPLC and lyophilization, 37 mg of PEG dimer was recovered. The theoretical yield for this experiment was 55 mg based on a calculated mass of 7600 mg/mmol for a yield of 68% (Table I).

EXAMPLE 5

PEG dimerization of peptide Ac-GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 20)

The modification of peptide (SEQ ID NO: 20) was carried out by resuspending 10.5 mg (0.0031 mmol) of polymer in 2.5 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 20) (0.0094 mmol, 20 mg, MW 2133) dissolved in 0.25 ml of 0.1% trifluoroacetic acid and the mixture incubated at 4°C for 28 hours. At that time, the reaction as monitored by HPLC was estimated to be approximately 30% complete, the temperature was shifted to ambient and an additional 27 hour incubation provided no net increase in product. Because of possible hydrolysis of the reactive polymer, an additional 5 mg of polymer was added and the incubation was continued for an additional 16 hours. At that time, 0.25 ml of 1 M tris-HCl, pH 7.5 was added and the reaction mix was incubated at 4°C for an additional 1 hour. The sample was subjected to analytical and preparative HPLC using a flatter gradient system as described in Example 8. The main preparative reaction product peak eluting at ca 48 minutes. After preparative HPLC and lyophilization, 10.4 mg of PEG dimer was recovered. The theoretical yield for this experiment was 34.4 mg based on a calculated mass of 7650 mg/mmol for a yield of 30% (Table I).

EXAMPLE 7

PEG dimerization of peptide (SEQ ID NO: 18)

The modification of peptide (SEQ ID NO: 18) was carried out by resuspending 1.2 mg (0.00036 mmol) of polymer in 0.5 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of the peptide (0.0011 mmol, 1.5 mg, MW 2177) dissolved in 0.05 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 20 hours. At that time, 0.1 ml of 1 M tris-HCl at pH 7.5 was added. The reaction mixture was incubated at 4°C for 1 hour. The sample was subjected to purification using an analytical HPLC system as described in Example 8. The main reaction product peak eluted at ca 38 minutes. After preparative HPLC and lyophilization, 1 mg of PEG dimer was recovered. The theoretical yield for this experiment was 2.2 mg based on a calculated mass of 6150 mg/mmol for a yield of 45% (Table I).

minutes. The major product peak eluting at 48 minutes was collected and lyophilized (Figure 2). These elution conditions were subsequently modified to improve the resolution of some conjugation products peptide (SEQ ID NO: 20), mPEG-peptide (SEQ ID NO: 8), peptide (SEQ ID NO: 14) from reaction by products. This was accomplished by application of a flatter linear gradient of 20-80% B over 60 minutes. The variation in retention time due to different peptides and elution condition is described as part of each synthesis example. The materials recovered from the main product peak from each reaction were subsequently analyzed by analytical reverse phase HPLC, MALDI-TOF mass spectrometry, EPO competitive binding potential and for in vitro bioactivity.

The activated PEG used in these experiments has an approximate molecular weight of 3400 and has amine reactive succinimidyl groups on either end of the difunctional linear polymer. This reactivity was employed to couple two equivalents of peptide (SEQ ID NO: 8) (MW= 2092) to the polymer with the concomitant liberation of two succinimidyl moieties resulting in a dimeric product as shown in Scheme I. Peptide (SEQ ID NO: 8) contains two potentially reactive amines, one at the N-terminus of the peptide and one in the side chain of the single lysine within the peptide sequence, so that a number of different connectivities between the two molecules was possible.

MALDI-TOF mass spectral analysis was supportive of the presence of the expected dimeric product (Figure 3) as indicated by a predominant species

EXAMPLE 9

EBP (EPO Binding Protein) Dimerization

This example demonstrates the interaction of peptide (SEQ ID NO: 8), peptide (SEQ ID NO: 16), peptide (SEQ ID NO: 18) and peptide (SEQ ID NO: 13) with EPO binding protein (EBP) using a bifunctional sulphydryl reactive crosslinker, (1,4-Di-[3'-(2'-pyridyldithio)propionamido]butane DPDPB.

To explore the interaction of peptide (SEQ ID NO: 8) with EBP, a bifunctional sulphydryl reactive crosslinker (DPDPB) was used in an attempt to stabilize a mimetic dependent dimeric structure. Control experiments demonstrated that the crosslinker does not inactivate the EPO binding potential of EBP or the proliferative properties of peptide (SEQ ID NO: 8). As shown in Figure 4, a dimeric EBP product was formed by co-incubation of the peptide, peptide (SEQ ID NO: 8), DPDPB and EBP. This data shows the ability of the peptide (SEQ ID NO: 8) to mediate formation of a soluble receptor dimer. To further explore this question, peptides (SEQ ID NO: 13), (SEQ ID NO: 16) and (SEQ ID NO: 18) were examined for their ability to mediate dimerization. As shown in Figure 4, lanes 7A and 8A, when peptide (SEQ ID NO: 13) was truncated at the carboxyl terminus, it retained good in vitro bioactivity and improved in vivo bioactivity, resulting in a crosslinking signal similar to peptide (SEQ ID NO: 8). However, peptide (SEQ ID NO: 18) did not appear to stabilize the dimerization signal (Figure 4, lanes 9A

EXAMPLE 10

IMMOBILIZED EBP BASED [^{125}I]EPO COMPETITION BINDING ASSAY

This study examined the binding capacity of the EPO PEG dimers to bind EPO receptors.

The extracellular domain of the human erythropoietin receptor (EPO binding protein, EBP) was expressed and overproduced in E. coli. As with many other recombinant eukaryotic proteins produced in E. coli, the protein appeared as an insoluble product in laboratory scale fermentations and was refolded and purified to obtain active protein. EPO binding protein produced by this method contains one free sulfhydryl group which can be modified without effecting the solution phase binding of ligand. In order to immobilize the EPO binding protein for equilibrium binding analysis and for competition binding assay, the EPO binding protein was covalently attached to agarose beads.

The iodoacetyl activation chemistry of Sufolink beads (Pierce Chemical Co, Rockford, IL) is specific for free thiols and assures that the linkage is not easily reversible. EBP-Sulfolink beads were made as follows: SulfoLink gel suspension (10 ml) was mixed with of coupling buffer (40 ml: 50 mM Tris, pH 8.3, 5 mM EDTA) and the gel was allowed to settle. The supernatant was removed and the EPO binding protein (0.3-1 mg/ml in coupling buffer) to be bound was added directly to the washed beads. The mixture was rocked

washes were passed through the pipet tip and collected for determination of the free EPO concentration. Equilibrium binding analysis of the specific association of [^{125}I]EPO with EPO mimetic binding proteins immobilized on these agarose beads indicates a Kd of 5 nM ± 2 based on a linear transformation (Scatchard) of the binding isotherm (Figure 5).

Competitive binding analysis assays of candidate peptides and dimer peptides were performed as outlined below. Individual peptides were dissolved in DMSO to prepare a stock solution 1 mM. Dimer peptides were contained within PBS at a concentration of 5 mM. All reaction tubes (in duplicate) contained 50 μL of EBP beads, 0.5 nM [^{125}I]EPO and 0-500 μM peptide in a total of 500 μL binding buffer.

The final concentration of DMSO was adjusted to 2.5% in all peptide assay tubes. At this concentration DMSO has no detectable effect since an examination of the sensitivity of the assay to DMSO demonstrated that concentrations of up to 25% DMSO (V/V) had no deleterious effect on binding. Non-specific binding was measured in each individual assay by inclusion of tubes containing a large excess of unlabelled EPO (1000 nM). Initial assay points with no added peptide were included in each assay to determine total binding. Binding mixtures were incubated overnight at room temperature with gentle rocking. The beads were then collected using Micro-columns (Isolab, Inc.) and washed with 3 mL of wash buffer. The columns containing the washed beads were placed in 12 x 75 mm glass tubes and bound radioactivity levels determined in

TABLE II

Table II. Binding and Cell Proliferation Studies

id	Relative Binding*	EPO-ED_{50} (μM)*	
		murine receptor	truncated human receptor
). No. 8	1	0.1	0.09
ris inact.polymer	60	IA[2]	IA
q. I.D. No. 8 covalent dimer #1	4	0.01 (10X)	0.0015 (60X)
q. I.D. No. 8 covalent dimer #2	3	0.01 (10X)	0.002 (45X)
.D. No. 13	1.6	0.08	0.02
q. I.D. No. 13 covalent dimer	3	0.01 (8X)	0.002 (10X)
.D. No. 20 (N-acetyl)	4	0.03	0.06
q. I.D. No. 20 covalent dimer	12	0.2 (-7X)	0.05
.D. No. 14 (terminal NH_2)	0.6	0.1	0.08
q. I.D. No. 14 covalent dimer	-	0.006 (16X)	0.001 (80X)

* Amount required to achieve the half maximal level of EPO dependent proliferation.(11pM)
[1]Binding relative to Seq. I.D. No. 8
[2]As inactive
[3]Note that all peptides are cyclic and were analyzed as COOH terminal amides (-$CONH_2$)

After a 42 hr incubation at 37°C (ca. 2 cell doublings) 1μCi/well of [^{3}H] thymidine was added and the incubation continued for 6 hr at which time the cells were harvested and counted to assess [^{3}H]thymidine incorporation as a measure of cell proliferation. Results are expressed as the amount of peptide or dimer peptide necessary to yield one half of the maximal activity obtained with recombinant EPO.

As shown in Figure 5 and Table II, the initial lot of PEG-peptide (SEQ ID NO: 8) dimer demonstrated ED_{50} values of 0.01 μm and 0.0015 μm in EPO responsive cell lines containing the murine or human EPO receptor, respectively. In both cell lines, the parent peptide, peptide (SEQ ID NO: 8), demonstrated an ED_{50} of 0.1 μM, indicating an increase in potency of 10 fold in the murine receptor line and almost 60 fold in the human receptor containing cells. Thus, the dimer was clearly more potent in murine and human lines than the peptides themselves. This was confirmed by generation of a second synthesis lot of PEG-peptide (SEQ ID NO: 8) dimer which resulted in a 10 and 45 fold increase in potency in the murine and human lines, respectively. Polymer alone, which was inactivated by treatment with Tris-HCl, demonstrated no activity in the cell proliferation assay.

A second EPO mimetic peptide, peptide (SEQ ID NO: 13), with the sequence GGTYSCHFGPLTWVCKPQ, was also subjected to a similar PEG dimerization protocol as that described above for peptide (SEQ ID NO: 8). The dimer product of PEG-peptide (SEQ ID NO: 13) is also more active than the unconjugated parent compound (Table II).

EXAMPLE 12

To further examine the connectivity of the peptides of the present invention to PEG, peptide molecules, which contained only an internal lysine group were used peptide (SEQ ID NO: 8) analog acetylated at the N-terminus peptide (SEQ ID NO: 20) and a sequence analog peptide (SEQ ID NO: 14) which only had a reactive N-terminal amine were PEG dimerized. In vitro proliferation data of these compounds suggest that potential dimerization through the free amino terminus has the most profound effect on bioactivity giving rise to a species about 80 fold more active than the monomeric parent peptide (SEQ ID NO: 14) dimer. Conjugation through the lysine side chain had no real effect on activity peptide (SEQ ID NO: 20) as did mono-PEG or di-PEG conjugation (Table III). This data indicates that the creation of a head to head dimer (both peptides attached through the N-terminus) using a PEG linker greatly enhances the potency of EPO peptides and approaches a level almost two logs greater than the free parent peptide. Further, this effect was not observed upon simple covalent attachment of linear PEG to peptide (SEQ ID NO: 8) indicating that dimerization is a critical determinant for this increased activity.

EXAMPLE 13

Polycythemic Exhyposic Mouse Bioassay.

This study demonstrates the ability of peptide (SEQ ID NO: 8)/PEG-dimers to retain in vivo bioactivity. Peptides were assayed for in vivo activity in the polycythemic mouse bioassay adapted from the method described by Cotes and Bangham (1961), Nature 191: 1065-1067. BDF1 mice were allowed to acclimate to ambient conditions for 7-10 days. Body weights were determined for all animals. Low weight animals (<15 grams) were not used. Mice were introduced to hypobaric chambers with a 24 hour conditioning cycle consisting of 0.40% +/- 0.02 atm. for 18 hours followed by 6 hours at ambient pressure for a total of 14 days. Following the 14 day period, mice were placed in ambient pressure for 72 hours prior to dosing. Test samples or recombinant Human Erythropoietin (rHuEPO) standards were diluted in an assay vehicle consisting of Phosphate Buffered Saline (PBS)-0.1% Bovine Serum Albumin (BSA). Peptide sample stock solutions (excluding peptide dimers) were first solubilized in dimethyl sulfoxide (DMSO). Control groups included one group of vehicle alone, and one group of (DMSO) at final concentration of 1%.

Each dose group contained 10 mice. Mice were injected subcutaneously (scruff of neck) with 0.5 ml of the appropriate sample. Forty eight hours following the sample injection, the mice were administered an intraperitoneal injection of 0.2 ml of [^{59}Fe]

EXAMPLE 14

This example shows that an inactive truncation analog of peptide (SEQ ID NO: 8), which lacks the critical tyrosine peptide (SEQ ID NO: 18), (SCHFGPLTWVCK), can be converted to an agonist on the human EPO receptor cell line by PEG dimerization. In this experiment, a 10^{-5} M concentration of the parent peptide had no activity above background while the dimeric peptide exhibited a level of proliferation twice as many cpm as background. As shown in Figure 8, the peptide alone (open squares) did not induce proliferation of the EPO responsive cells but upon PEG dimerization (open diamonds) a significant agonist effect was observed. Approximately twice as many cpm incorporated over non-stimulated cells at 10^{-5} M added peptide dimer. The replicate error bars represent the standard deviation of three assay points per concentration of peptide or peptide dimer.

refrigerated centrifuge. The supernatant was removed, the cell pellet resuspended in 100 µl of binding buffer, and the cell suspension layered onto 0.7 ml of bovine calf serum. The tubes were centrifuged at 12,000 rpm for 5 min at 4°C, the supernatant was removed, the bottom of the tubes snipped off, and the cell pellets counted in a Micromedic ME plus gamma counter. Non-specific binding was determined by incubating cells with [125]-EPO and a 100-fold excess of non-radioactive EPO. These data demonstrate increases in apparent binding competitive affinity of 3.0 fold, 3.2 fold and 80 fold for peptides RWJ 61233, RWJ 61596 and RWJ 61718, respectively (Table 2). In vivo proliferation studies with these peptides and their dimer derivatives reveal increases in potency of ea. 50 fold, 10 fold and 80 fold, respectively, indicating that the magnitude of increased binding affinity is exceeded by the functional potency of the peptide for two of the three species. Thus, the effect of dimerization and subsequent increase in activity may be one in which the efficiency of receptor stimulation is improved by limiting the lateral diffusion of the receptors away from a binding event. Peptide dimerization therefore likely results in entropic rather than enthalpic gains upon mimetic ligand-receptor association for some peptide dimer sequences.

Unlike the EBP-bead EPO competitive binding assay where peptide dimerization negatively impacted the ability of PEG dimer peptides to compete for receptor binding, the ability to compete for cell associated receptors is increased by dimerization. This may be due to the ability of the cell associated receptor to dimerize while the immobilized EBP monomer likely cannot.

Conversion of inactive to active peptide RWJ 61177 was further studied. An improved and expanded study was performed which confirmed our earlier observation of conversion to an active peptide (Figure 6, Panel D).

TABLE V. EPO COMPETITIVE BINDING ANALYSIS OF CELL ASSOCIATED RECEPTORS

Compound	IC_{50} (μM)
RWJ 61233 (seq ID #8)	3.8
SAP2/61233, covalent dimer	0.28
RWJ 61718 (seq ID#14)	18
SAP2/61718, covalent dimer	0.07

c) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..10
(D) OTHER INFORMATION: /note= "Xaa(Pos1) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos2) can be R,H,L or W; Xaa(Pos3) can be M,F or I; Xaa(Pos6) can be any one of the 20 L-amino acids or the stereoisomeric D-amino acids; Xaa(Pos9) can be D,E,I,L or V; and Xaa(Pos10) can be C,A,α-amino-γ-bromobutyric acid or Hoc, provided that either Xaa(Pos1) or Xaa(Pos10) is C or Hoc"

i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

aa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa
5 10

NFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Xaa(Pos2) and Xaa(Pos8) can be any one of the 20 L-amino acids; Xaa(Pos3) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos4) can be R,H,L or W; Xaa(Pos5) can be M,F or I; Xaa(Pos11) can be D,E,I,L or V; and Xaa(Pos12) can be C,A,α-amino-γ-bromobutyric acid or Hoc, provided that either Xaa(Pos3) or Xaa(Pos12) is C or Hoc"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr Xaa Xaa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa
1 5 10

INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

aa Tyr Xaa Cys Xaa Xaa Gly Pro Xaa Thr Trp Xaa Cys Xaa Xaa Xaa
5 10 15

FORMATION FOR SEQ ID NO:6:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

.x) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Xaa(Pos1) can be D,E,L,N,S,T or V; Xaa(Pos3) can be A,H,K,L,M,S or T; Xaa(Pos5) can be R or H; Xaa(Pos6) can be F or M; Xaa(Pos9) can be I,L,T,M or V; Xaa(Pos12) can be D or V; Xaa(Pos14) can be K,R,S or T; Xaa(Pos15) is P and Xaa(Pos16) can be any one of the 20 L-amino acids"

xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Xaa Tyr Xaa Cys Xaa Xaa Gly Pro Xaa Thr Trp Xaa Cys Xaa Xaa Xaa
1 5 10 15

NFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Gly Leu Tyr Leu Cys Arg Phe Gly Pro Val Thr Trp Asp Cys Gly
1 5 10 15

Tyr Lys Gly Gly
20

(i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Gly Asn Tyr Met Cys His Phe Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

NFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gly Gly Val Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Val Gly Asn Tyr Met Ala His Met Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly

INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid

FORMATION FOR SEQ ID NO:16:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

hr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10

FORMATION FOR SEQ ID NO:17:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Tyr Cys His Phe Gly Pro Leu Thr Trp Val Cys
1 5 10

NFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
1 5 10

INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ly Gly Thr Tyr Arg Cys Ser Met Gly Pro Met Thr Trp Val Cys Leu
5 10 15

ro Met Gly Gly
20

FORMATION FOR SEQ ID NO:22:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gly Gly Met Tyr Ser Cys Arg Met Gly Pro Met Thr Trp Val Cys Gly
1 5 10 15

Pro Ser Gly Gly
20

NFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Gly Gly Trp Ala Trp Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Ala His Gly Gly
20

i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ly Gly Asp Tyr Thr Cys Arg Met Gly Pro Met Thr Trp Ile Cys Thr
5 10 15

la Thr Gly Gly
20

FORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Gly Gly Asn Tyr Leu Cys Arg Phe Gly Pro Gly Thr Trp Asp Cys Thr
1 5 10 15

Gly Phe Arg Gly
20

NFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Gly Gly Asn Tyr Val Cys Arg Met Gly Pro Ile Thr Trp Ile Cys Thr
1 5 10 15

Pro Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

FORMATION FOR SEQ ID NO:32:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

;ly Gly Glu Tyr Lys Cys Arg Met Gly Pro Leu Thr Trp Val Cys Gln
1 5 10 15

Tyr Ala Gly Gly
20

NFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Gly Gly Asp Tyr Thr Cys Arg Met Gly Pro Met Thr Trp Ile Cys Thr
1 5 10 15

Ala Thr Arg Gly
20

INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

ly Gly Asn Tyr Ile Cys Arg Met Gly Pro Met Thr Trp Val Cys Thr
5 10 15

la His Gly Gly
20

FORMATION FOR SEQ ID NO:38:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Gly Gly Asp Tyr Leu Cys Arg Met Gly Pro Ala Thr Trp Val Cys Gly
1 5 10 15

Arg Met Gly Gly
20

NFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Gly Gly Leu Tyr Leu Cys Arg Phe Gly Pro Val Thr Trp Asp Cys Gly
1 5 10 15

Tyr Lys Gly Gly
20

) SEQUENCE DESCRIPTION: SEQ ID NO:42:

y Gly Thr Tyr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10 15

:o Gln Gly Gly
20

:ORMATION FOR SEQ ID NO:43:

:) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:43:

:ly Gly Ile Tyr Lys Cys Leu Met Gly Pro Leu Thr Trp Val Cys Thr
5 10 15

>ro Asp Gly Gly
20

NFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Gly Gly Leu Tyr Ser Cys Leu Met Gly Pro Ile Thr Trp Leu Cys Lys
1 5 10 15

Pro Lys Gly Gly
20

:NFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

FORMATION FOR SEQ ID NO:48:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Gly Gly Lys Tyr Leu Cys Ser Phe Gly Pro Ile Thr Trp Val Cys Ala
1 5 10 15

Arg Tyr Gly Gly
20

NFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Gly Gly Leu Tyr Lys Cys Arg Leu Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Gly Gly Leu Tyr Glu Cys Arg Met Gly Pro Met Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

NFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Gly Gly Asp Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Ile Cys Thr
1 5 10 15

Lys Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Gly Gly Val Tyr Ser Cys Arg Met Gly Pro Thr Thr Trp Glu Cys Asn
1 5 10 15

Arg Tyr Val Gly
20

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:61:

ly Gly Val Tyr Lys Cys Arg Met Gly Pro Leu Thr Trp Glu Cys Arg
5 10 15

ro Thr Gly Gly
20

FORMATION FOR SEQ ID NO:62:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Gly Gly Asp Tyr Asn Cys Arg Phe Gly Pro Leu Thr Trp Val Cys Lys
1 5 10 15

Pro Ser Gly Gly
20

NFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Gly Gly Ser Tyr Leu Cys Arg Phe Gly Pro Thr Thr Trp Leu Cys Ser
1 5 10 15

Ser Ala Gly Gly
20

i) SEQUENCE DESCRIPTION: SEQ ID NO:66:

ly Gly Trp Val Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Gly
5 10 15

al His Gly Gly
20

FORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

Gly Gly Gln Leu Leu Cys Gly Ile Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Trp Val Gly Gly
20

NFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

Gly Gly Lys Tyr Ser Cys Phe Met Gly Pro Thr Thr Trp Val Cys Ser
1 5 10 15

Pro Val Gly Arg Gly Val
20

INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

FORMATION FOR SEQ ID NO:80:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

<i) SEQUENCE DESCRIPTION: SEQ ID NO:80:

Gly Gly Lys Tyr Tyr Cys Trp Met Gly Pro Met Thr Trp Val Cys Ser
1 5 10 15

Pro Ala Gly Gly
20

NFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

Gly Gly Tyr Val Met Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Asp
1 5 10 15

Ile Pro Gly Gly
20

INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

Gly Gly Leu Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Lys
1 5 10 15

Tyr Met Ala Gly
20

NFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

Gly Gly Gln Tyr Leu Cys Thr Phe Gly Pro Ile Thr Trp Leu Cys Arg
1 5 10 15

Gly Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

Gly Gly Val Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

.) SEQUENCE DESCRIPTION: SEQ ID NO:90:

:y Gly Asn Tyr Tyr Cys Arg Phe Gly Pro Ile Thr Phe Glu Cys His
5 10 15

ro Thr Gly Gly
20

FORMATION FOR SEQ ID NO:91:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:91:

Gly Gly Glu Tyr Leu Cys Arg Met Gly Pro Asn Thr Trp Val Cys Thr
1 5 10 15

Pro Val Gly Gly
20

NFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Gly Gly Leu Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Leu
1 5 10 15

Pro Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

Gly Gly Trp Val Tyr Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Asp
1 5 10 15

Thr Asn Gly Gly
20

NFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

Gly Gly Met Tyr Tyr Cys Arg Met Gly Pro Met Thr Trp Val Cys Lys
1 5 10 15

Gly Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

Gly Gly Thr Thr Gln Cys Trp Ile Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Ala Arg Gly Gly
20

) SEQUENCE DESCRIPTION: SEQ ID NO:74:

y Gly Asn Tyr Thr Cys Arg Phe Gly Pro Leu Thr Trp Glu Cys Thr
5 10 15

o Gln Gly Gly Gly Ala
20

'ORMATION FOR SEQ ID NO:75:

.) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:75:

ly Gly Ser Trp Asp Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Lys
5 10 15

rp Ser Gly Gly
20

FORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

Val Gly Asn Tyr Met Cys His Phe Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

NFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

agonist, insulin agonist, IL-3 agonist, IL-5 agonist, IL-6 agonist or IL-2 agonist.

20. The method of Claim 15 or 16 wherein said agonist comprises a sequence of amino acids $YX_2X_3X_4X_5GPX_6TWX_7X_8$, (SEQ ID NO: 2) wherein each of X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

21. The method of Claim 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein each of X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

22. The method of Claim 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1Y_1X_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein each of X_1, X_2 and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R or H; X_5 is F or M; X_6 is I, L, T, M or V; X_7 is D or V; X_9 is G, K, L, Q, R, S, or T; and X_{10} is A, G, P, R, or Y.

23. The method of Claims 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein X_1 is D, E, L, N, S, T or V; X_2 is A, H, K, L, M, S, or T; X_3 is C; X_4 is R or H; X_5 is M, F or I; X_6 and X_{11} are independently any one of the 20 genetically coded L-

29. The method of Claim 28 wherein said antagonist comprises a sequence of amino acids $(AX_2)_nX_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 19) wherein X_6 is selected from any of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; X_8 is C; X_2 is selected from any of the 20 genetically coded L-amino acids, n is 0 or 1 and A is any of the 20 genetically coded L-amino acids except Y (tyrosine).

30. The method of Claim 21 where said antagonist is SCHFGPLTWVCK (SEQ ID NO: 18).

Preparative Reverse Phase Analysis of PEG- Peptide (SEQ. I.D. No. 8) Dimer

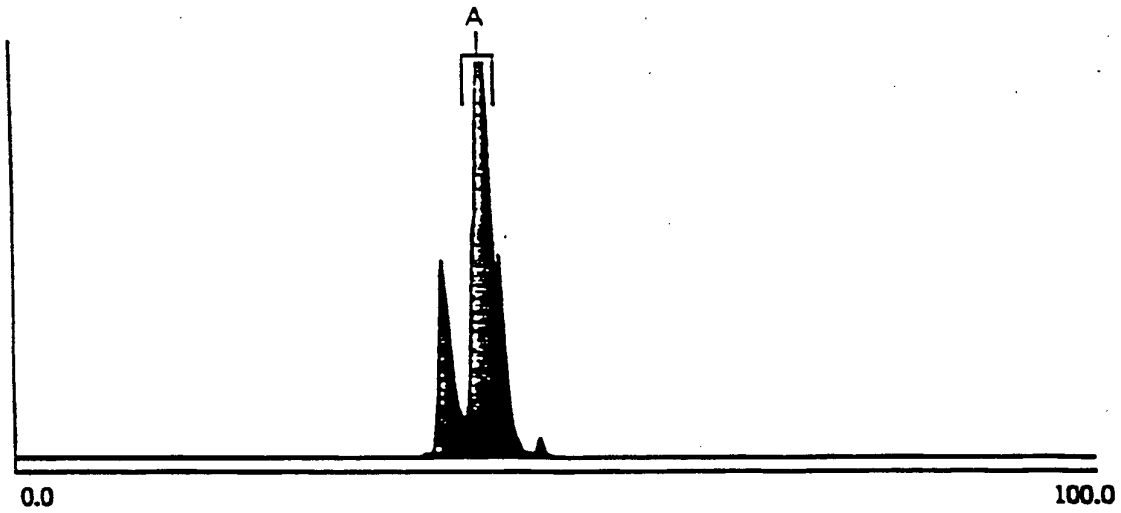
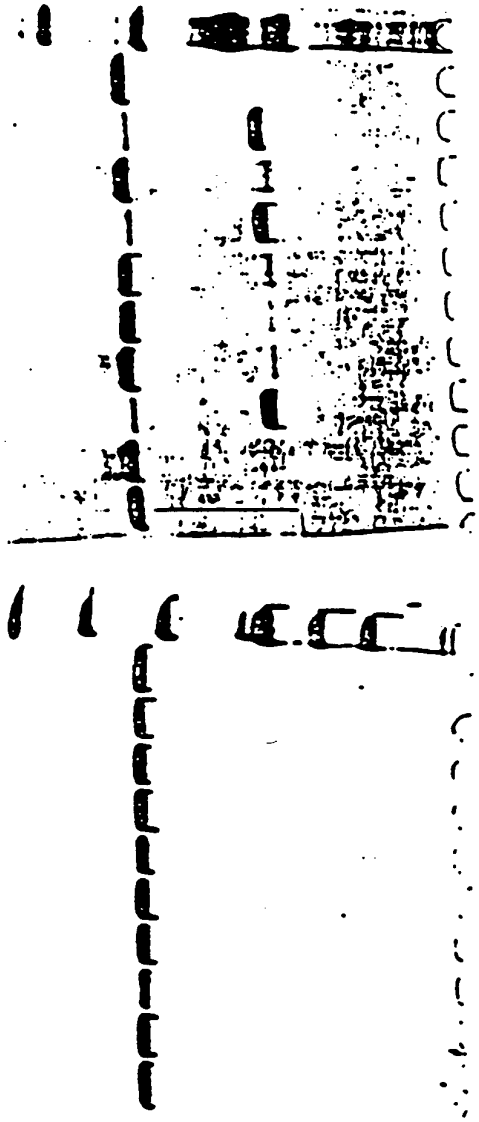


Figure 2



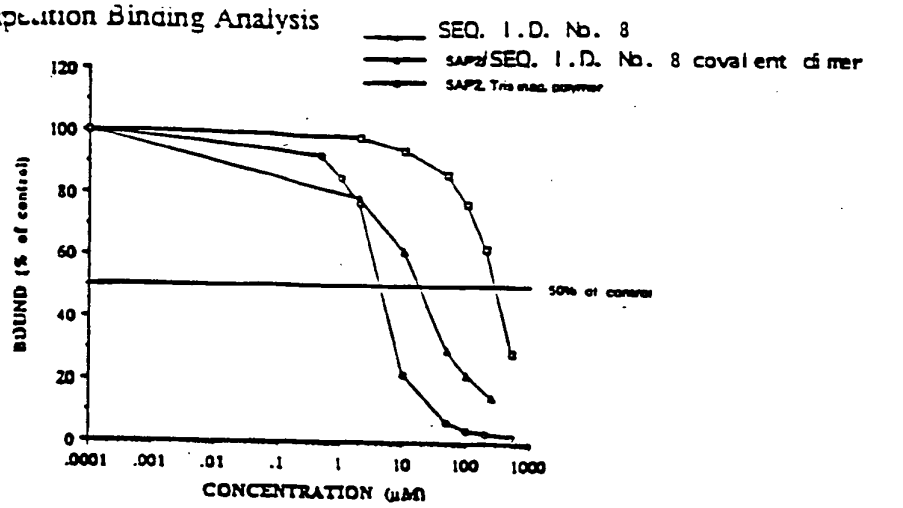
A. Non Reducing 10-20% SDS-PAGE. B. Reducing 10-20% SDS-PAGE

Lane	1	2	Seq. I.D. No.	8		16		18		13	
MWM	EBP	Peptide (μM)	400	400	400	400	400	400	400	400	...
	EBP (μM)		22	22	22	22	22	22	22	22	22
	DPDTG (mM)		1.1	0	1.1	0	1.1	0	1.1	0	1.1

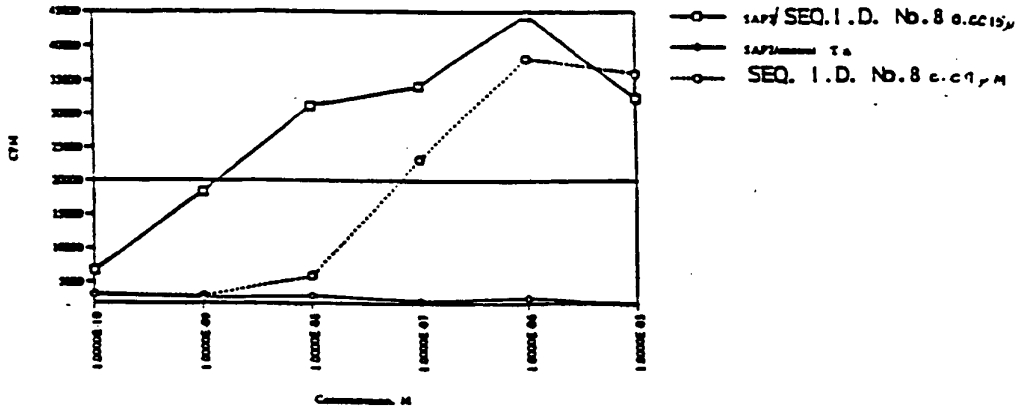
Seq. I.D. No.	Sequence	IC_{50} (μM)	EPO-ED_{50} (μM)
8	GGTYSCHFGPLTWVCKPQGG	5	0.1
16	YSCHFGPLTWVCK	70	3
18	SCHFGPLTWVCK	90	IA
13	GGTYSCHFGPLTWVCKPQ	8	0.08

Figure 4

A. [125I]EPO Competition Binding Analysis



B. Cell Proliferation, Human EPOR



C. Cell Proliferation, Murine EPOR

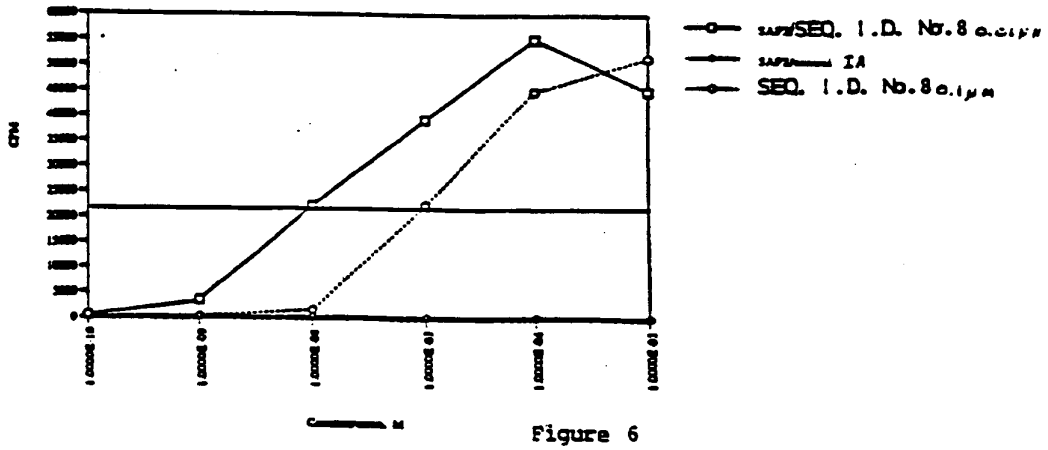


Figure 6

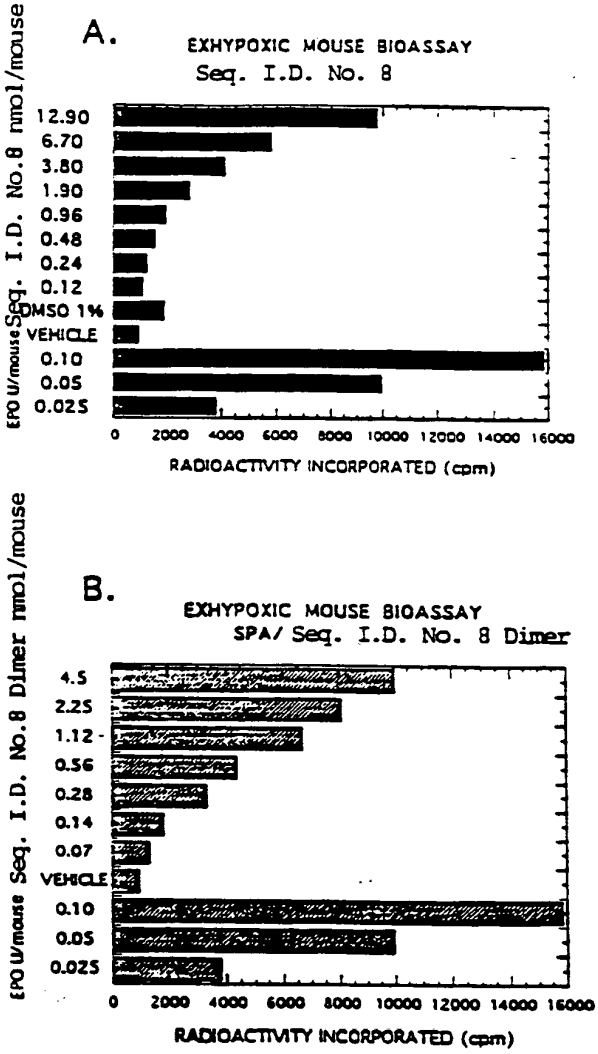


Figure 7

FIGURE 9

Page 1 of 3

Ac-GGTYSCHFGPLTWVCKPQGG SEQ ID NO. 20

[illegible] SEQ ID NO: 21

GGNYSCRMGPNTWVCGPSGG SEQ ID NO: 22

GGNAWCRMGPITNVCSAHGG SEQ ID NO: 23

GGNYSCRMGPITNVCGPYGG SEQ ID NO: 24

GGEYRCRMGPITNVCKPEGG SEQ ID NO: 25

GGDYTCRMGPNTWICDAGGG SEQ ID NO: 26

GGNYLCPFGPGTNDCTSPRG SEQ ID NO: 27

GGNYVCRMGPITWICTPAGG SEQ ID NO: 28

GGRDVCRMGPITNDCRSTGG SEQ ID NO: 29

GGSYLCRMGPTTNLCTAQRGGG SEQ ID NO: 30

GGNYLCRMGPATNVCGRMGG SEQ ID NO: 31

GGEYRCRMGPLTNVCGYAGG SEQ ID NO: 32

GGDYTCRMGPNTWICDARRG SEQ ID NO: 33

GGVYVCRMGPLTNECDRSGG SEQ ID NO: 34

GGEYSCRMGPNTWVCSPTGG SEQ ID NO: 35

GGEYLCRMGPITNVCERYGG SEQ ID NO: 36

GGNYICRMGPMTWVCTAHGG SEQ ID NO: 37

GGDYLCRMGPATWVCGRMGG SEQ ID NO: 38

GGLYLCRFGPVTWDCGYNGG SEQ ID NO: 39

GGLYSCRMGPITWVCTKAGG SEQ ID NO: 40

GGGYHCRMGPMTWVCRPVGG SEQ ID NO: 41

GGTYSCHFGPLTWVCKPQGG SEQ ID NO: 42

GGIYRCLMGPLTNVCTPDGG SEQ ID NO: 43

GGLYSCLMGPITNLCKPNGG SEQ ID NO: 44

GGDYHCRMGPLTWVCKPLGG SEQ ID NO: 45

GGDYSCRMGPTTNVCTPPGG SEQ ID NO: 46

GGDYWCRMGPSTWECNAHGG SEQ ID NO: 47

GGKYLCSFGPITNVCARYGG SEQ ID NO: 48

GGLYRCRLGPITWVCSPLGG SEQ ID NO: 49

GGSYTCRFGPEIWVCRPNGG SEQ ID NO: 50

GGSYSCRMGPITWVCKPGGG SEQ ID NO: 51

GGSYTCRMGPITWVCLPAGG SEQ ID NO: 52

GGLYSCRMGPHTWVCRPGGG SEQ ID NO: 53

Figure 9
Page 3 of 3

GGEYLCRMGPMTTNCTPVGG SEQ ID NO: 91
GGLYTCRMGPITTVCLPAGG SEQ ID NO: 92
GGLYTCRMGPITTVCLPAGG SEQ ID NO: 93

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 14/505, A61K 38/18

A2

(11) International Publication Number: WO 96/40772

(43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/US96/09469

(22) International Filing Date: 6 June 1996 (06.06.96)

(30) Priority Data:
08/484,135 7 June 1995 (07.06.95) US

(71) Applicant *(for all designated States except US)*: JOHNSON & JOHNSON [US/US]; One Johnson & Johnson Plaza, New Brunswick, NJ 08933-0001 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: JOHNSON, Dana, L. [US/US]; 1343 Lonely Cottage Road, Upper Black Eddy, PA 18972 (US). ZIVIN, Robert, A. [US/US]; 6 Glenbrook Court, Lawrenceville, NJ 08648 (US).

(74) Agents: CIAMPORCERO, Audley, A., Jr. et al.; Johnson & Johnson, One Johnson & Johnson Plaza, New Brunswick, NJ 08933-0001 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: AGONIST PEPTIDE DIMERS

(57) Abstract

The present invention is directed to the dimerization of agonists and antagonists of cell surface receptors and particularly to peptide dimers which behave as cell surface receptor agonists in their dimeric form. Such receptors belong to the dimerization-mediated activation class often observed among receptors for growth and differentiation factors. The agonists of this class of receptors is understood to effect dimerization of the receptor and thus signal initiation. The present invention exemplifies dimers of erythropoietin (EPO) agonists and antagonists comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid or Hoc; X_4 can be R, H, L or W; X_5 can be M, F, or I; X_6 is idependently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid or Hoc, provided that either X_3 or X_8 is C or Hoc.

* (Referred to in PCT Gazette No. 07/1997, Section II)

BNSDOCID: <WO___9640772A2_IA>

AGONIST PEPTIDE DIMERS

FIELD OF THE INVENTION

The present invention is directed to the dimerization of agonists and antagonists of cell surface receptors and particularly to peptide dimers which behave as cell surface receptor agonists in their dimeric form. Such receptors belong to the dimerization-mediated activation class often observed among receptors for growth and differentiation factors. The agonists of this class of receptors is understood to effect dimerization of the receptor and thus signal initiation. The present invention exemplifies dimers of erythropoietin (EPO) agonists and antagonists comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid or Hoc; X_4 can be R, H, L or W; X_5 can be M, F, or I; X_6 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid or Hoc, provided that either X_3 or X_8 is C or Hoc.

BACKGROUND OF THE INVENTION

Erythropoietin (EPO) is a glycoprotein hormone with an approximate molecular weight of 34,000 daltons. The primary role of EPO, which is synthesized in the kidneys of mammals, is to stimulate mitotic cell division and differentiation of erythrocyte precursor cells. As a result, EPO acts to stimulate and to

Claus-Walker et al. (1984) Arch. Phys. Med. Rehabil. 65:370-374); space flight (See, Dunn et al. (1984) Eur. J. Appl. Physiol. 52:178-182); acute blood loss (See, Miller et al. (1982) Brit. J. Haematol. 52:545-590); aging (See, Udupa et al. (1984) J. Lab. Clin. Med. 103:574-588); various neoplastic disease states accompanied by abnormal erythropoiesis (See, Dainiak et al. (1983) Cancer 5:1101-1106); and renal insufficiency (See, Eschbach et al. (1987) N. Eng. J. Med. 316:73-78).

Although purified, homogenous EPO has been characterized, little is known about the mechanism of EPO-induced erythroblast proliferation and differentiation. The specific interaction of EPO with progenitor cells of immature red blood cells, platelets, and megakaryocytes has not been described. This is due in part, to the small number of surface EPO receptor molecules on normal erythroblasts and on the erythroleukemia cell lines. See Krantz and Goldwasser (1984) Proc. Natl. Acad. Sci. USA, 81:7574-7578; Branch et al. (1987) Blood 69:1782-1785; Mayeux et al. (1987) FEBS Letters 211:229-223; Mufson and Gesner (1987) Blood 69:1485-1490; Sakaguchi et al. (1987) Biochem. Biophys. Res. Commun. 146:7-12; Sawyer et al. (1987) Proc. Natl. Acad. Sci. USA 84:3690-3694; Sawyer et al. (1987) J. Biol. Chem. 262:5554-5562; and Todokoro et al. (1988) Proc. Natl. Acad. Sci. USA 84:4126-4130. The DNA sequences and encoded peptide sequences for murine and human EPO receptor proteins have been described. See, D'Andrea et al. PCT Patent Publication No. WO 90/08822 (published 1990).

dimers have two 'monomeric' peptide units of 10 to 40 or more amino acids, preferably 14 to about 20 residues in length, comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) where each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_6 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc. Preferably, the monomeric peptide unit of the dimer comprises a core sequence $YX_2X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 2) where each amino acid is indicated by standard one letter abbreviation; each X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

More preferably, the monomeric peptide unit of the dimer comprises a core sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3), where each amino acid is indicated by standard one letter abbreviation; each X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is

SCHFGPLTWVCK (SEQ ID NO: 18).

Other particularly preferred monomeric peptide units of the present dimers include peptides comprising a core sequence of the formula $(AX_2)_nX_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 19) wherein X_2 through X_8 are as previously defined herein (SEQ ID NO: 2), n is 1 or 0 and A is any one of the naturally occurring L-amino acids except Y (tyrosine); n is defined herein as the number of occurrences of (AX_2) which can be 1 or none in the core sequence. When (AX_2) is present, i.e. when n = 1, A is not tyrosine and A is not any non-naturally occurring aromatic amino acid analog. Such monomeric peptide units of the dimers of this invention can be prepared by truncating the peptides of Fig. 9, for example, from the N-terminus to delete the Y, tyrosine residue in SEQ ID NOS. 21 - 93. Such monomeric peptides can also be prepared by substitution of Y in position A in the peptides of Fig. 9.

In accordance with the present invention the monomeric units of the dimers can be the same or different.

In a preferred embodiment polyethylene glycol (PEG) is employed as a linker to form the dimeric peptides of the present invention through a covalent bond.

In another embodiment, the present invention is directed to pharmaceutical compositions comprising at least one dimer peptide of the invention and a pharmaceutical carrier.

Fig. 2 shows a major peak, with a retention time of 48 minutes, following purification of the dimerized EPO peptide, (SEQ ID NO: 8).

Fig. 3 depicts the MALDI-TOF mass spectral analysis of the dimerized peptides, including peptide (SEQ ID NO: 8), GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13) and SCHFGPLTWVCK (SEQ ID NO: 18).

Fig. 4 shows the SDS-PAGE analysis of DPDPB crosslinking of EPO binding protein (EBP) in the presence and absence of EPO agonist peptides.

Fig. 5 demonstrates equilibrium EPO binding to immobilized EPO binding protein. Panel A represents the equilibrium binding data and Panel B (inset) is the linear transformation (Scatchard) of the data set in Panel A.

Fig. 6 depicts the results of a competitive binding assay run on the EPO agonist peptide (SEQ ID NO: 8) in competitive binding with [^{125}I]EPO to EBP beads (Panel A); and EPO responsive cell proliferation studies in FDC-P1 derived cell lines containing either a human (Panel B) or murine EPO receptor (Panel C).

Fig. 7 is a graphic representation of the results of the exhypoxic mouse bioassay; stimulation of the incorporation of [^{59}Fe] into nascent red blood cells by EPO, peptide (SEQ ID NO: 8) (Panel A) and peptide (SEQ ID NO: 8) dimer (Panel B).

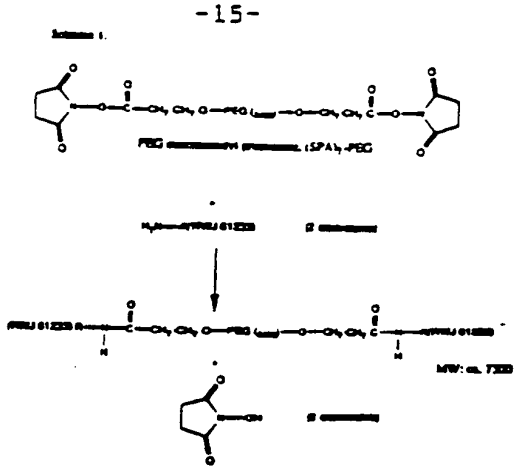
Fig. 8 demonstrates the effect of PEG dimerization of peptide (SEQ ID NO: 18) activity in EPO responsive cell proliferation studies in FDC-P1 derived cell lines containing a human EPO receptor.

cell-surface receptors in vitro and in vivo. Such receptors include, for example, EPO, GM-CSF, G-CSF, M-CSF, GH, EGF, PDGF, VEGF, Insulin and FGF. Other receptors which are activated by heterodimerization or multimerization may also be subject to activation by this mechanism including, IL-3, IL-5, IL-6, IL-2 and TPO. The dimers of the present invention have two 'monomeric' peptide units of 10 to 40 or more amino acids, preferably 14 to about 20 amino acid residues in length. In a preferred embodiment, these monomeric peptide units comprise a core sequence of amino acids $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) where each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_6 is independently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc. Preferably, the monomeric peptide unit of the dimer comprises a core sequence $YX_2X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 2) where each amino acid is indicated by standard one letter abbreviation; each X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine; X_4 can be R, H, L, or W; X_5 can be M, F, or I; X_7 can be D, E, I, L, or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid, or Hoc, where Hoc is homocysteine, provided that either X_3 or X_8 is C or Hoc.

GGDYHCRMGPLTWVCKPLGG (SEQ ID NO: 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID NO: 10);
GGVYACRMGPITWVCSPLGG (SEQ ID NO: 11);
VGNYMAHMGPITWVCRPGG (SEQ ID NO: 12);
GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13);
GGLYACHMGPMTWVCQPLRG (SEQ ID NO: 14);
TIAQYICYMGPETWECRPSPKA (SEQ ID NO: 15);
YSCHFGPLTWVCK (SEQ ID NO: 16);
YCHFGPLTWVC (SEQ ID NO: 17); and
SCHFGPLTWVCK (SEQ ID NO: 18).

The dimer peptides of the present invention exhibit increased biological potency in vitro and in vivo relative to the monomeric agonists from which the dimers are derived. Moreover, cell surface receptor antagonists can be 'converted' to cell surface receptor agonists in accordance with the present invention. Specifically, a cell surface receptor antagonist can be dimerized with PEG or another appropriate linker which permits mutual binding of the monomeric moieties with the receptors. As a result, the dimer exhibits effective binding to the target receptor and behaves as an agonist. Accordingly, the dimers of this invention demonstrate enhanced biological potency in vitro and in vivo relative to their monomeric forms.

The dimer peptides of the present invention bind to and biologically activate the cell surface receptor or otherwise behave as agonists and are preferably formed by employing polyethylene glycol as a linker between the monomeric peptide units described herein. While other conventional chemical systems can



Dimerization and especially pegylation in a head-to-head (amino to amino terminus) or head-to-tail (amino to carboxyl terminus) configuration is preferred relative to internal covalent binding of the monomeric peptides. The 'monomer' units of the dimer peptides of the present invention can be the same or different, although the same are preferred.

The monomeric peptides which are used to form the dimers of the present invention can be prepared by classical chemical methods well known in the art. The standard methods include, for example, exclusive solid phase synthesis and recombinant DNA technology. See, e.g. Merrifield (1963) J. Am. Chem. Soc. 85:2149. Solid phase synthesis is typically commenced from the C-terminal end of the peptide using an α-amino protected resin. A suitable starting material can be prepared by attaching the required α-amino acid to a chloromethylated resin (such as BIO-BEADS SX-1, Bio Rad Laboratories, Richmond, CA), a hydroxymethyl resin, (described by Bodonszky et al. (1966) Chem. Ind.

Boc. The side chain protecting groups for Lys include Cbz, 2-chlorobenzyloxycarbonyl (2-Cl-Cbz), 2-bromobenzyloxycarbonyl (2-BrCbz), Tos, or Boc.

After removal of the α-amino protecting group, the remaining protected amino acids are coupled stepwise in the desired order. Each protected amino acid is generally reacted in about a 3-fold excess using an appropriate carboxyl group activator such as 2-(1H-benxotriazol-1-yl)-1,1,3,3tetramethyluronium hexafluorophosphate (HBTU) or dicyclohexylcarbodiimide (DCC) in solution of methylene chloride (CH_2CI_2), or dimethyl formamide (DMF) mixtures.

After the desired amino acid sequence has been completed, the desired peptide is decoupled from the resin support by treating the mixture with a reagent such as trifluoroacetic acid (TFA) or hydrogen fluoride (HF). These reagents not only cleave the peptide from the resin, but also cleave all remaining side chain protecting groups. When the chloromethylated resin is used, hydrogen fluoride treatment results in the formation of the free peptide acids. When the benzhydrylamine resin is used, hydrogen fluoride treatment results directly in the free peptide amide. Alternatively, when the chloromethylated resin is employed, the side chain protected peptide can be decoupled by treatment of the peptide resin with ammonia to give the desired side chain protected amide or with an alkylamine to give a side chain protected alkylamide or dialkylamide. Side chain protection is then removed in the usual fashion by treatment with hydrogen fluoride to give the free amides, alkylamides, or dialkylamides.

flexibility leading to fewer barriers to effective receptor interaction and subsequently receptor activation. This is also indicated for molecules which can bind but not activate a receptor subtype in that such molecules can become more effective inhibitors of ligand binding.

The present invention further provides a method for altering a cell-surface receptor antagonist, a molecule exhibiting receptor binding but no biological activity, to behave as a cell-surface receptor agonist in vitro or in vivo. This method is achieved by dimerizing the antagonist molecule with an appropriate linker molecule such as PEG, other polymerized molecules or a peptide. In a preferred embodiment, an EPO antagonist, i.e. a peptide exhibiting receptor binding but no biological EPO activity can be altered by dimerization to obtain a dimer which behaves as an EPO receptor agonist. Thus, for example, in the case of EPO-R these include the monomeric peptide units of the present dimers comprising a core sequence of general formula $(AX_2)_nX_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 19) wherein X_2 through X_8 are as previously defined herein, in (SEQ ID NO: 2), n is 1 or 0 and A is any one of the naturally occurring L-amino acids except Y (tyrosine); n is defined herein as the number of occurrences of (AX_2) which can be 1 or none in the core sequence. When X_2 is present, i.e., when n = 1, A is not tyrosine and A is not any non-naturally occurring aromatic amino acid analog. Such monomeric peptide units of the dimers of this invention can be prepared by truncating the peptides of Fig. 9, for example, from the N-terminus to

iodohydroxyphenylalanine, p-fluorohydroxyphenylalanine, p-amino-hydroxyphenylalanine act as EPO-R monomer agonists but substitution with threonine or alanine for tyrosine at position Y causes the monomer peptide to act as an EPO-R antagonist. However, when dimerized in accordance with the present invention, such dimers behave as EPO-R agonists. The monomeric peptide units identified at Fig. 9, for example, behave as EPO-R antagonists in the absence of tyrosine at position Y of the formula above. When such antagonists are dimerzied, the dimer behaves as an EPO-R agonist.

In a further embodiment of the present invention, pharmaceutical compositions comprising at least one of the dimers of this invention can be employed to therapeutically treat disorders resulting from deficiencies of biological factors such as EPO, GH, GM-CSF, G-CSF, EGF, PDGF, VEGF, insulin, FGF and the like. These pharmaceutical compositions may contain buffers, salts and other excipients to stabilize the composition or assist in the delivery of the dimerized molecules.

In a preferred embodiment, the present invention provides a method for treating disorders associated with a deficiency of EPO. The method is accomplished by administering at least one of the dimers identified herein for a time and under conditions sufficient to alleviate the symptoms of the disorder, i.e. sufficient to effect dimerization or biological activation of EPO receptors. In the case of EPO such methodology is useful in the treatment of end-stage renal failure/dialysis; anemia, especially associated

(nasal, vaginal, rectal, or sublingual) routes of administration in dosage forms appropriate for each route of administration.

Solid dosage forms for oral administration include capsules, tablets, pill, powders, and granules. In such solid dosage forms, the active compound is admixed with at least one inert pharmaceutically acceptable carrier such as sucrose, lactose, or starch. Such dosage forms can also comprise, as it normal practice, additional substances other than inert diluents, e.g., lubricating, agents such as magnesium stearate. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering, agents. Tablets and pills can additionally be prepared with enteric coatings.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, with the elixirs containing inert diluents commonly used in the art, such as water. Besides such inert diluents, compositions can also include adjuvants, such as wetting agents, emulsifying and suspending agents, and sweetening, flavoring and perfuming agents.

Preparations according to this invention for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting,

EXAMPLE 1

SDS-PAGE gels (10-20% gradient SDS-PAG plates, 84 x 70 x 1.0 mm, Integrated Separation Systems, Natick, MA) were stained with Coomasie Brilliant Blue R-250 (BioRad). A commercial preparation of activated difunctional polyethylene glycol (PEG-succinimidyl-propionate, SPA2, MW ca. 3400) was purchased from Shearwater Polymers, Huntsville, AL as was the monofunctional reagent, methoxy-PEG-succinimidyl-proprionate, MW ca 5000. Peptide (SEQ ID NO: 8) and all other peptides were obtained from the Peptide Synthesis Facility RWJ-PRI, La Jolla, CA or Quality Controlled Biochemical, Hopkinton MA. These peptides were cyclized via oxidation of their intramolecular cysteines, amidated at the C-terminus and mass confirmed by FAB-MS. All were Ellman Reaction negative. Tris base was obtained from BioRad, Hercules, CA. (DPDPB) and trifluoracetic acid (HPLC grade) were obtained from Pierce Chemical Co., Rockford IL.

Mono-PEG conjugation of peptide GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8)

This example describes the preparation of mono-PEG conjugates of peptide (SEQ ID NO: 8), using the monofunctional amine reactive polymer analog m-SPA-PEG to be used as a control in experiments described herein. The reaction was carried out with polymer in excess (ca. 3 fold) by resuspending 142.5 mg (0.0286 mmol, MW ca. 5000) of polymer in 4 ml PBS at pH 7.5 and adding 20 mg peptide (SEQ ID NO: 8) (0.0095 mmol, MW 2092) dissolved

TABLE 1

Recovery Yield of Peptide Conjugation Reaction and Apparent Molecular Mass of Product

I.D. No.	Sequence	Mass	Conjugation Reagent	Main Product Mass (centroid m/z)	Yield (% of theoretical)
8	GGTYSCHFGPLTWVCKPQGG	2092	SPA2-PEG (MW ca. 3400)	7834	69
			m-SPA-PEG (MW ca. 5000)	7092 (peak 1) 12036 (peak 2)	- -
13	GGTYSCHFGPLTWVCKPQ	1978	SPA2-PEG	7560	54
20	Ac-GGTYSCHFGPLTWVCKPQGG	2133	SPA2-PEG	7862	30
14	GGLYACHMGPMTWVCQPLRG	2177	SPA2-PEG	7872	37
18	SCHFGPLTWVCK	1375	SPA2-PEG	6326	45

EXAMPLE 3

PEG dimerization of peptide GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8) (lot #2)

The modification of peptide (SEQ ID NO: 8) was carried out by resuspending 25 mg (0.0071 mmol) of polymer in 4 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 8) (0.0213 mmol, 45.8 mg, MW 2092) dissolved in 1 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 22 hours. At that time, the reaction was adjusted to a final concentration of 50 mM Tris by the addition of 1 M tris-HCl, pH 7.5. The reaction mixture was incubated on ice for 1 hour. The sample was subjected to analytical and preparative HPLC as described in Example 8. After preparative HPLC and lyophilization, 37 mg of PEG dimer was recovered. The theoretical yield for this experiment was 55 mg based on a calculated mass of 7600 mg/mmol for a yield of 68% (Table I).

EXAMPLE 5

PEG dimerization of peptide Ac-GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 20)

The modification of peptide (SEQ ID NO: 20) was carried out by resuspending 10.5 mg (0.0031 mmol) of polymer in 2.5 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of peptide (SEQ ID NO: 20) (0.0094 mmol, 20 mg, MW 2133) dissolved in 0.25 ml of 0.1% trifluoroacetic acid and the mixture incubated at 4°C for 28 hours. At that time, the reaction as monitored by HPLC was estimated to be approximately 30% complete, the temperature was shifted to ambient and an additional 27 hour incubation provided no net increase in product. Because of possible hydrolysis of the reactive polymer, an additional 5 mg of polymer was added and the incubation was continued for an additional 16 hours. At that time, 0.25 ml of 1 M tris-HCl, pH 7.5 was added and the reaction mix was incubated at 4°C for an additional 1 hour. The sample was subjected to analytical and preparative HPLC using a flatter gradient system as described in Example 8. The main preparative reaction product peak eluting at ca 48 minutes. After preparative HPLC and lyophilization, 10.4 mg of PEG dimer was recovered. The theoretical yield for this experiment was 34.4 mg based on a calculated mass of 7650 mg/mmol for a yield of 30% (Table I).

EXAMPLE 7

PEG dimerization of peptide (SEQ ID NO: 18)

The modification of peptide (SEQ ID NO: 18) was carried out by resuspending 1.2 mg (0.00036 mmol) of polymer in 0.5 ml PBS at pH 7.5, and adding a ca. 3 fold molar excess of the peptide (0.0011 mmol, 1.5 mg, MW 2177) dissolved in 0.05 ml of 0.1% trifluoroacetic acid. The mixture was incubated on ice for 20 hours. At that time, 0.1 ml of 1 M tris-HCl at pH 7.5 was added. The reaction mixture was incubated at 4°C for 1 hour. The sample was subjected to purification using an analytical HPLC system as described in Example 8. The main reaction product peak eluted at ca 38 minutes. After preparative HPLC and lyophilization, 1 mg of PEG dimer was recovered. The theoretical yield for this experiment was 2.2 mg based on a calculated mass of 6150 mg/mmol for a yield of 45% (Table I).

minutes. The major product peak eluting at 48 minutes was collected and lyophilized (Figure 2). These elution conditions were subsequently modified to improve the resolution of some conjugation products peptide (SEQ ID NO: 20), mPEG-peptide (SEQ ID NO: 8), peptide (SEQ ID NO: 14) from reaction by products. This was accomplished by application of a flatter linear gradient of 20-80% B over 60 minutes. The variation in retention time due to different peptides and elution condition is described as part of each synthesis example. The materials recovered from the main product peak from each reaction were subsequently analyzed by analytical reverse phase HPLC, MALDI-TOF mass spectrometry, EPO competitive binding potential and for in vitro bioactivity.

The activated PEG used in these experiments has an approximate molecular weight of 3400 and has amine reactive succinimidyl groups on either end of the difunctional linear polymer. This reactivity was employed to couple two equivalents of peptide (SEQ ID NO: 8) (MW= 2092) to the polymer with the concomitant liberation of two succinimidyl moieties resulting in a dimeric product as shown in Scheme I. Peptide (SEQ ID NO: 8) contains two potentially reactive amines, one at the N-terminus of the peptide and one in the side chain of the single lysine within the peptide sequence, so that a number of different connectivities between the two molecules was possible.

MALDI-TOF mass spectral analysis was supportive of the presence of the expected dimeric product (Figure 3) as indicated by a predominant species

EXAMPLE 9

EBP (EPO Binding Protein) Dimerization

This example demonstrates the interaction of peptide (SEQ ID NO: 8), peptide (SEQ ID NO: 16), peptide (SEQ ID NO: 18) and peptide (SEQ ID NO: 13) with EPO binding protein (EBP) using a bifunctional sulphydryl reactive crosslinker, (1,4-Di-[3'-(2'-pyridyldithio)propionamido]butane DPDPB.

To explore the interaction of peptide (SEQ ID NO: 8) with EBP, a bifunctional sulphydryl reactive crosslinker (DPDPB) was used in an attempt to stabilize a mimetic dependent dimeric structure. Control experiments demonstrated that the crosslinker does not inactivate the EPO binding potential of EBP or the proliferative properties of peptide (SEQ ID NO: 8). As shown in Figure 4, a dimeric EBP product was formed by co-incubation of the peptide, peptide (SEQ ID NO: 8), DPDPB and EBP. This data shows the ability of the peptide (SEQ ID NO: 8) to mediate formation of a soluble receptor dimer. To further explore this question, peptides (SEQ ID NO: 13), (SEQ ID NO: 16) and (SEQ ID NO: 18) were examined for their ability to mediate dimerization. As shown in Figure 4, lanes 7A and 8A, when peptide (SEQ ID NO: 13) was truncated at the carboxyl terminus, it retained good *in vitro* bioactivity and improved *in vivo* bioactivity, resulting in a crosslinking signal similar to peptide (SEQ ID NO: 8). However, peptide (SEQ ID NO: 18) did not appear to stabilize the dimerization signal (Figure 4, lanes 9A

EXAMPLE 10

IMMOBILIZED EBP BASED [^{125}I]EPO COMPETITION BINDING ASSAY

This study examined the binding capacity of the EPO PEG dimers to bind EPO receptors.

The extracellular domain of the human erythropoietin receptor (EPO binding protein, EBP) was expressed and overproduced in E. coli. As with many other recombinant eukaryotic proteins produced in E. coli, the protein appeared as an insoluble product in laboratory scale fermentations and was refolded and purified to obtain active protein. EPO binding protein produced by this method contains one free sulfhydryl group which can be modified without effecting the solution phase binding of ligand. In order to immobilize the EPO binding protein for equilibrium binding analysis and for competition binding assay, the EPO binding protein was covalently attached to agarose beads.

The iodoacetyl activation chemistry of Sufolink beads (Pierce Chemical Co, Rockford, IL) is specific for free thiols and assures that the linkage is not easily reversible. EBP-Sulfolink beads were made as follows: SulfoLink gel suspension (10 ml) was mixed with of coupling buffer (40 ml: 50 mM Tris, pH 8.3, 5 mM EDTA) and the gel was allowed to settle. The supernatant was removed and the EPO binding protein (0.3-1 mg/ml in coupling buffer) to be bound was added directly to the washed beads. The mixture was rocked

washes were passed through the pipet tip and collected for determination of the free EPO concentration. Equilibrium binding analysis of the specific association of [^{125}I]EPO with EPO mimetic binding proteins immobilized on these agarose beads indicates a Kd of 5 nM ± 2 based on a linear transformation (Scatchard) of the binding isotherm (Figure 5).

Competitive binding analysis assays of candidate peptides and dimer peptides were performed as outlined below. Individual peptides were dissolved in DMSO to prepare a stock solution 1 mM. Dimer peptides were contained within PBS at a concentration of 5 mM. All reaction tubes (in duplicate) contained 50 µL of EBP beads, 0.5 nM [^{125}I]EPO and 0-500 µM peptide in a total of 500 µL binding buffer.

The final concentration of DMSO was adjusted to 2.5% in all peptide assay tubes. At this concentration DMSO has no detectable effect since an examination of the sensitivity of the assay to DMSO demonstrated that concentrations of up to 25% DMSO (V/V) had no deleterious effect on binding. Non-specific binding was measured in each individual assay by inclusion of tubes containing a large excess of unlabelled EPO (1000 nM). Initial assay points with no added peptide were included in each assay to determine total binding. Binding mixtures were incubated overnight at room temperature with gentle rocking. The beads were then collected using Micro-columns (Isolab, Inc.) and washed with 3 mL of wash buffer. The columns containing the washed beads were placed in 12 x 75 mm glass tubes and bound radioactivity levels determined in

TABLE II

Table II. Binding and Cell Proliferation Studies

Id	Relative Binding[1]	EPO-ED_{50} (μM)[2]	
		murine receptor	truncated human receptor
D. No. 8	1	0.1	0.09
ris inact.polymer	60	IA[3]	IA
q. I.D. No. 8 covalent dimer #1	4	0.01 (10X)	0.0015 (60X)
q. I.D. No. 8 covalent dimer #2	3	0.01 (10X)	0.002 (45X)
D. No. 13	1.6	0.08	0.02
q. I.D. No. 13 covalent dimer	3	0.01 (8X)	0.002 (10X)
D. No. 20 (N-acetyl)	4	0.03	0.06
q. I.D. No. 20 covalent dimer	12	0.2 (-7X)	0.05
D. No. 14 (terminal NH_2)	0.6	0.1	0.08
q. I.D. No. 14 covalent dimer	-	0.006 (16X)	0.001 (80X)

[1] Amount required to achieve the half maximal level of EPO dependent proliferation (1 pM)
[2] Binding relative to Seq. I.D. No. 8
[3] As is assay
[4] Note that all peptides are cyclic and were analyzed as COOH terminal amides (-$CONH_2$)

After a 42 hr incubation at 37°C (ca. 2 cell doublings) 1μCi/well of [^{3}H] thymidine was added and the incubation continued for 6 hr at which time the cells were harvested and counted to assess [^{3}H]thymidine incorporation as a measure of cell proliferation. Results are expressed as the amount of peptide or dimer peptide necessary to yield one half of the maximal activity obtained with recombinant EPO.

As shown in Figure 5 and Table II, the initial lot of PEG-peptide (SEQ ID NO: 8) dimer demonstrated ED_{50} values of 0.01 μm and 0.0015 μm in EPO responsive cell lines containing the murine or human EPO receptor, respectively. In both cell lines, the parent peptide, peptide (SEQ ID NO: 8), demonstrated an ED_{50} of 0.1 μM, indicating an increase in potency of 10 fold in the murine receptor line and almost 60 fold in the human receptor containing cells. Thus, the dimer was clearly more potent in murine and human lines than the peptides themselves. This was confirmed by generation of a second synthesis lot of PEG-peptide (SEQ ID NO: 8) dimer which resulted in a 10 and 45 fold increase in potency in the murine and human lines, respectively. Polymer alone, which was inactivated by treatment with Tris-HCl, demonstrated no activity in the cell proliferation assay.

A second EPO mimetic peptide, peptide (SEQ ID NO: 13), with the sequence GGTYSCHFGPLTWVCKPQ, was also subjected to a similar PEG dimerization protocol as that described above for peptide (SEQ ID NO: 8). The dimer product of PEG-peptide (SEQ ID NO: 13) is also more active than the unconjugated parent compound (Table II).

EXAMPLE 12

To further examine the connectivity of the peptides of the present invention to PEG, peptide molecules, which contained only an internal lysine group were used peptide (SEQ ID NO: 8) analog acetylated at the N-terminus peptide (SEQ ID NO: 20) and a sequence analog peptide (SEQ ID NO: 14) which only had a reactive N-terminal amine were PEG dimerized. In vitro proliferation data of these compounds suggest that potential dimerization through the free amino terminus has the most profound effect on bioactivity giving rise to a species about 80 fold more active than the monomeric parent peptide (SEQ ID NO: 14) dimer. Conjugation through the lysine side chain had no real effect on activity peptide (SEQ ID NO: 20) as did mono-PEG or di-PEG conjugation (Table III). This data indicates that the creation of a head to head dimer (both peptides attached through the N-terminus) using a PEG linker greatly enhances the potency of EPO peptides and approaches a level almost two logs greater than the free parent peptide. Further, this effect was not observed upon simple covalent attachment of linear PEG to peptide (SEQ ID NO: 8) indicating that dimerization is a critical determinant for this increased activity.

EXAMPLE 13

Polycythemic Exhyposic Mouse Bioassay.

This study demonstrates the ability of peptide (SEQ ID NO: 8)/PEG-dimers to retain in vivo bioactivity. Peptides were assayed for in vivo activity in the polycythemic mouse bioassay adapted from the method described by Cotes and Bangham (1961), Nature 191: 1065-1067. BDF1 mice were allowed to acclimate to ambient conditions for 7-10 days. Body weights were determined for all animals. Low weight animals (<15 grams) were not used. Mice were introduced to hypobaric chambers with a 24 hour conditioning cycle consisting of 0.40% +/- 0.02 atm. for 18 hours followed by 6 hours at ambient pressure for a total of 14 days. Following the 14 day period, mice were placed in ambient pressure for 72 hours prior to dosing. Test samples or recombinant Human Erythropoietin (rHuEPO) standards were diluted in an assay vehicle consisting of Phosphate Buffered Saline (PBS)-0.1% Bovine Serum Albumin (BSA). Peptide sample stock solutions (excluding peptide dimers) were first solubilized in dimethyl sulfoxide (DMSO). Control groups included one group of vehicle alone, and one group of (DMSO) at final concentration of 1%.

Each dose group contained 10 mice. Mice were injected subcutaneously (scruff of neck) with 0.5 ml of the appropriate sample. Forty eight hours following the sample injection, the mice were administered an intraperitoneal injection of 0.2 ml of [^{59}Fe]

EXAMPLE 14

This example shows that an inactive truncation analog of peptide (SEQ ID NO: 8), which lacks the critical tyrosine peptide (SEQ ID NO: 18), (SCHFGPLTWVCK), can be converted to an agonist on the human EPO receptor cell line by PEG dimerization. In this experiment, a 10^{-3} M concentration of the parent peptide had no activity above background while the dimeric peptide exhibited a level of proliferation twice as many cpm as background. As shown in Figure 8, the peptide alone (open squares) did not induce proliferation of the EPO responsive cells but upon PEG dimerization (open diamonds) a significant agonist effect was observed. Approximately twice as many cpm incorporated over non-stimulated cells at 10^{-3} M added peptide dimer. The replicate error bars represent the standard deviation of three assay points per concentration of peptide or peptide dimer.

refrigerated centrifuge. The supernatant was removed, the cell pellet resuspended in 100 µl of binding buffer, and the cell suspension layered onto 0.7 ml of bovine calf serum. The tubes were centrifuged at 12,000 rpm for 5 min at 4°C, the supernatant was removed, the bottom of the tubes snipped off, and the cell pellets counted in a Micromedic ME plus gamma counter. Non-specific binding was determined by incubating cells with [125]-EPO and a 100-fold excess of non-radioactive EPO. These data demonstrate increases in apparent binding competitive affinity of 3.0 fold, 3.2 fold and 80 fold for peptides RWJ 61233, RWJ 61596 and RWJ 61718, respectively (Table 2). In vivo proliferation studies with these peptides and their dimer derivatives reveal increases in potency of ea. 50 fold, 10 fold and 80 fold, respectively, indicating that the magnitude of increased binding affinity is exceeded by the functional potency of the peptide for two of the three species. Thus, the effect of dimerization and subsequent increase in activity may be one in which the efficiency of receptor stimulation is improved by limiting the lateral diffusion of the receptors away from a binding event. Peptide dimerization therefore likely results in entropic rather than enthalpic gains upon mimetic ligand-receptor association for some peptide dimer sequences.

Unlike the EBP-bead EPO competitive binding assay where peptide dimerization negatively impacted the ability of PEG dimer peptides to compete for receptor binding, the ability to compete for cell associated receptors is increased by dimerization. This may be due to the ability of the cell associated receptor to dimerize while the immobilized EBP monomer likely cannot.

Conversion of inactive to active peptide RWJ 61177 was further studied. An improved and expanded study was performed which confirmed our earlier observation of conversion to an active peptide (Figure 6, Panel D).

TABLE V. EPO COMPETITIVE BINDING ANALYSIS OF CELL ASSOCIATED RECEPTORS

Compound	IC_{50} (μM)
RWJ 61233 (seq ID #8)	3.8
SAP2/61233, covalent dimer	0.28
RWJ 61718 (seq ID#14)	18
SAP2/61718, covalent dimer	0.07

c) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..10
(D) OTHER INFORMATION: /note= "Xaa(Pos1) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos2) can be R,H,L or W; Xaa(Pos3) can be M,F or I; Xaa(Pos6) can be any one of the 20 L-amino acids or the stereoisomeric D-amino acids; Xaa(Pos9) can be D,E,I,L or V; and Xaa(Pos10) can be C,A,α-amino-γ-bromobutyric acid or Hoc, provided that either Xaa(Pos1) or Xaa(Pos10) is C or Hoc"

i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

aa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa
5 10

FORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..12
(D) OTHER INFORMATION: /note= "Xaa(Pos2) and Xaa(Pos8) can be any on of the 20 L-amino acids; Xaa(Pos3) can be C,A,α-amino-γ-bromobutyric acid or Hoc; Xaa(Pos4) can be R,H,L or W; Xaa(Pos5) can be M,F or I; Xaa(Pos11) can be D,E,I,L or V; and Xaa(Pos12) can be C,A,α-amino-γ-bromobutyric acid or Hoc, provided that either Xaa(Pos3) or Xaa(Pos12) is C or Hoc"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Tyr Xaa Xaa Xaa Xaa Gly Pro Xaa Thr Trp Xaa Xaa
1 5 10

INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

aa Tyr Xaa Cys Xaa Xaa Gly Pro Xaa Thr Trp Xaa Cys Xaa Xaa Xaa
5 10 15

FORMATION FOR SEQ ID NO:6:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

.x) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..16
(D) OTHER INFORMATION: /note= "Xaa(Pos1) can be D,E,L,N,S,T or V; Xaa(Pos3) can be A,H,K,L,M,S or T; Xaa(Pos5) can be R or H; Xaa(Pos6) can be F or M; Xaa(Pos9) can be I,L,T,M or V; Xaa(Pos12) can be D or V; Xaa(Pos14) can be K,R,S or T; Xaa(Pos15) is P and Xaa(Pos16) can be any one of the 20 L-amino acids"

xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Xaa Tyr Xaa Cys Xaa Xaa Gly Pro Xaa Thr Trp Xaa Cys Xaa Xaa Xaa
1 5 10 15

NFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Gly Gly Leu Tyr Leu Cys Arg Phe Gly Pro Val Thr Trp Asp Cys Gly
1 5 10 15

Tyr Lys Gly Gly
20

(i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Gly Asn Tyr Met Cys His Phe Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Gly Gly Val Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Val Gly Asn Tyr Met Ala His Met Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly

INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid

FORMATION FOR SEQ ID NO:16:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

hr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10

FORMATION FOR SEQ ID NO:17:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Tyr Cys His Phe Gly Pro Leu Thr Trp Val Cys
1 5 10

NFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
1 5 10

INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ly Gly Thr Tyr Arg Cys Ser Met Gly Pro Met Thr Trp Val Cys Leu
5 10 15

ro Met Gly Gly
20

FORMATION FOR SEQ ID NO:22:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gly Gly Met Tyr Ser Cys Arg Met Gly Pro Met Thr Trp Val Cys Gly
1 5 10 15

Pro Ser Gly Gly
20

NFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Gly Gly Trp Ala Trp Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Ala His Gly Gly
20

i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ly Gly Asp Tyr Thr Cys Arg Met Gly Pro Met Thr Trp Ile Cys Thr
5 10 15

la Thr Gly Gly
20

FORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Gly Gly Asn Tyr Leu Cys Arg Phe Gly Pro Gly Thr Trp Asp Cys Thr
1 5 10 15

Gly Phe Arg Gly
20

NFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Gly Gly Asn Tyr Val Cys Arg Met Gly Pro Ile Thr Trp Ile Cys Thr
1 5 10 15

Pro Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

FORMATION FOR SEQ ID NO:32:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Gly Gly Glu Tyr Lys Cys Arg Met Gly Pro Leu Thr Trp Val Cys Gln
1 5 10 15

Tyr Ala Gly Gly
20

NFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Gly Gly Asp Tyr Thr Cys Arg Met Gly Pro Met Thr Trp Ile Cys Thr
1 5 10 15

Ala Thr Arg Gly
20

INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:37:

ly Gly Asn Tyr Ile Cys Arg Met Gly Pro Met Thr Trp Val Cys Thr
5 10 15

la His Gly Gly
20

FORMATION FOR SEQ ID NO:38:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Gly Gly Asp Tyr Leu Cys Arg Met Gly Pro Ala Thr Trp Val Cys Gly
1 5 10 15

Arg Met Gly Gly
20

NFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Gly Gly Leu Tyr Leu Cys Arg Phe Gly Pro Val Thr Trp Asp Cys Gly
1 5 10 15

Tyr Lys Gly Gly
20

) SEQUENCE DESCRIPTION: SEQ ID NO:42:

y Gly Thr Tyr Ser Cys His Phe Gly Pro Leu Thr Trp Val Cys Lys
5 10 15

:o Gln Gly Gly
20

'ORMATION FOR SEQ ID NO:43:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:43:

ly Gly Ile Tyr Lys Cys Leu Met Gly Pro Leu Thr Trp Val Cys Thr
5 10 15

ro Asp Gly Gly
20

NFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Gly Gly Leu Tyr Ser Cys Leu Met Gly Pro Ile Thr Trp Leu Cys Lys
1 5 10 15

Pro Lys Gly Gly
20

:NFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

FORMATION FOR SEQ ID NO:48:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Gly Gly Lys Tyr Leu Cys Ser Phe Gly Pro Ile Thr Trp Val Cys Ala
1 5 10 15

Arg Tyr Gly Gly
20

NFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Gly Gly Leu Tyr Lys Cys Arg Leu Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Gly Gly Leu Tyr Glu Cys Arg Met Gly Pro Met Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

NFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Gly Gly Asp Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Ile Cys Thr
1 5 10 15

Lys Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Gly Gly Val Tyr Ser Cys Arg Met Gly Pro Thr Thr Trp Glu Cys Asn
1 5 10 15

Arg Tyr Val Gly
20

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:61:

ly Gly Val Tyr Lys Cys Arg Met Gly Pro Leu Thr Trp Glu Cys Arg
5 10 15

ro Thr Gly Gly
20

FORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

Gly Gly Asp Tyr Asn Cys Arg Phe Gly Pro Leu Thr Trp Val Cys Lys
1 5 10 15

Pro Ser Gly Gly
20

NFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

Gly Gly Ser Tyr Leu Cys Arg Phe Gly Pro Thr Thr Trp Leu Cys Ser
1 5 10 15

Ser Ala Gly Gly
20

i) SEQUENCE DESCRIPTION: SEQ ID NO:66:

ly Gly Trp Val Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Gly
5 10 15

al His Gly Gly
20

NFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

Gly Gly Gln Leu Leu Cys Gly Ile Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Trp Val Gly Gly
20

INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

Gly Gly Lys Tyr Ser Cys Phe Met Gly Pro Thr Thr Trp Val Cys Ser
1 5 10 15

Pro Val Gly Arg Gly Val
20

INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

FORMATION FOR SEQ ID NO:80:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

<i) SEQUENCE DESCRIPTION: SEQ ID NO:80:

Gly Gly Lys Tyr Tyr Cys Trp Met Gly Pro Met Thr Trp Val Cys Ser
1 5 10 15

Pro Ala Gly Gly
20

NFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

Gly Gly Tyr Val Met Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Asp
1 5 10 15

Ile Pro Gly Gly
20

INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

Gly Gly Leu Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Lys
1 5 10 15

Tyr Met Ala Gly
20

NFORMATION FOR SEQ ID NO:86:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

Gly Gly Gln Tyr Leu Cys Thr Phe Gly Pro Ile Thr Trp Leu Cys Arg
1 5 10 15

Gly Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:87:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

Gly Gly Val Tyr Ala Cys Arg Met Gly Pro Ile Thr Trp Val Cys Ser
1 5 10 15

Pro Leu Gly Gly
20

.) SEQUENCE DESCRIPTION: SEQ ID NO:90:

ly Gly Asn Tyr Tyr Cys Arg Phe Gly Pro Ile Thr Phe Glu Cys His
5 10 15

ro Thr Gly Gly
20

FORMATION FOR SEQ ID NO:91:

i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

.i) MOLECULE TYPE: peptide

:i) SEQUENCE DESCRIPTION: SEQ ID NO:91:

Gly Gly Glu Tyr Leu Cys Arg Met Gly Pro Asn Thr Trp Val Cys Thr
1 5 10 15

Pro Val Gly Gly
20

NFORMATION FOR SEQ ID NO:92:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

Gly Gly Leu Tyr Thr Cys Arg Met Gly Pro Ile Thr Trp Val Cys Leu
1 5 10 15

Pro Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:93:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

Gly Gly Trp Val Tyr Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Asp
1 5 10 15

Thr Asn Gly Gly
20

NFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

Gly Gly Met Tyr Tyr Cys Arg Met Gly Pro Met Thr Trp Val Cys Lys
1 5 10 15

Gly Ala Gly Gly
20

INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

Gly Gly Thr Thr Gln Cys Trp Ile Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Ala Arg Gly Gly
20

) SEQUENCE DESCRIPTION: SEQ ID NO:74:

y Gly Asn Tyr Thr Cys Arg Phe Gly Pro Leu Thr Trp Glu Cys Thr
5 10 15

o Gln Gly Gly Gly Ala
20

'ORMATION FOR SEQ ID NO:75:

.) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

i) MOLECULE TYPE: peptide

i) SEQUENCE DESCRIPTION: SEQ ID NO:75:

ly Gly Ser Trp Asp Cys Arg Ile Gly Pro Ile Thr Trp Val Cys Lys
5 10 15

rp Ser Gly Gly
20

IFORMATION FOR SEQ ID NO:76:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

ii) MOLECULE TYPE: peptide

xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

Val Gly Asn Tyr Met Cys His Phe Gly Pro Ile Thr Trp Val Cys Arg
1 5 10 15

Pro Gly Gly Gly
20

:NFORMATION FOR SEQ ID NO:77:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid

GGLYLCRFGPVTWDCGYKGG (SEQ ID NO: 7);
GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8);
GGDYHCRMGPLTWVCKPLGG (SEQ ID NO: 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID NO: 10);
GGVYACRMGPITWVCSPLGG (SEQ ID NO: 11);
VGNYMAHMGPITWVCRPGG (SEQ ID NO: 12);
GGTYSCHFGPLTWVCKPQ (SEQ ID NO: 13);
GGLYACHMGPMTWVCQPLRG (SEQ ID NO: 14);
TIAQYICYMGPETWECRPSPKA (SEQ ID NO: 15);
YSCHFGPLTWVCK (SEQ ID NO: 16);
YCHFGPLTWVC (SEQ ID NO: 17); and
SCHFGPLTWVCK (SEQ ID NO: 18)

7. A pharmaceutical composition comprising at least one peptide dimer of any one of Claims 1-6.

8. A method for treating a patient having a disorder characterized by a deficiency of EPO or low or defective red blood cell population comprising administering to said patient a therapeutically effective amount of at least one peptide dimer of any one of Claims 1-6.

9. The peptide dimer of any one of Claims 1-6 wherein said dimer is formed by a polyethylene glycol linker through a covalent bond.

10. The peptide dimer of any one of Claims 1-6 wherein said monomeric peptide units are dimerized on activated benodiazepins, oxazalones, azalactones, aminimides or diketopiperazine.

11. The peptide dimer of Claim 9 wherein said monomeric peptides are covalently bound N-terminus to N-terminus.

wherein each of X_2 and X_6 is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

21. The method of Claim 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein each of X_1, X_2, X_6, X_9, X_{10}, and X_{11} is independently selected from any one of the 20 genetically coded Lamino acids; X_3 is C; X_4 is R, H, L or W; X_5 is M, F or I; X_7 is D, E, I, L or V; and X_8 is C.

22. The method of Claim 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein each of X_1, X_2, and X_{11}, is independently selected from any one of the 20 genetically coded L-amino acids; X_3 is C; X_4 is R or H; X_5 is F or M; X_6 is I, L, T, M or V; X_7 is D or V; X_9 is G, K, L, Q, R, S, or T; and X_{10} is A, G, P, R, or Y.

23. The method of Claims 15 or 16 wherein said agonist comprises a sequence of amino acids $X_1YX_2X_3X_4X_5GPX_6TWX_7X_8X_9X_{10}X_{11}$ (SEQ ID NO: 3) wherein X_1 is D, E, L, N, S, T or V; X_2 is A, H, K, L, M, S, or T; X_3 is C; X_4 is R or H; X_5 is M, F or I; X_6 and X_{11} are independently any one of the 20 genetically coded L-amino acids; X_7 is D, E, I, L or V; X_8 is C; X_9 is K, R, S, or T; and X_{10} is P.

24. The method of Claim 15 or 16 wherein said agonist is selected from the group consisting of:

GGLYLCRFGPVTWDCGYKGG (SEQ ID NO: 7);
GGTYSCHFGPLTWVCKPQGG (SEQ ID NO: 8);
GGDYHCRMGPLTWVCKPLGG (SEQ ID NO: 9);
VGNYMCHFGPITWVCRPGGG (SEQ ID NO: 10);
GGVYACRMGPITWVCSPLGG (SEQ ID NO: 11);

Reverse Phase Analysis of SPA2 Reaction with SEQ. I.D. NO. 8

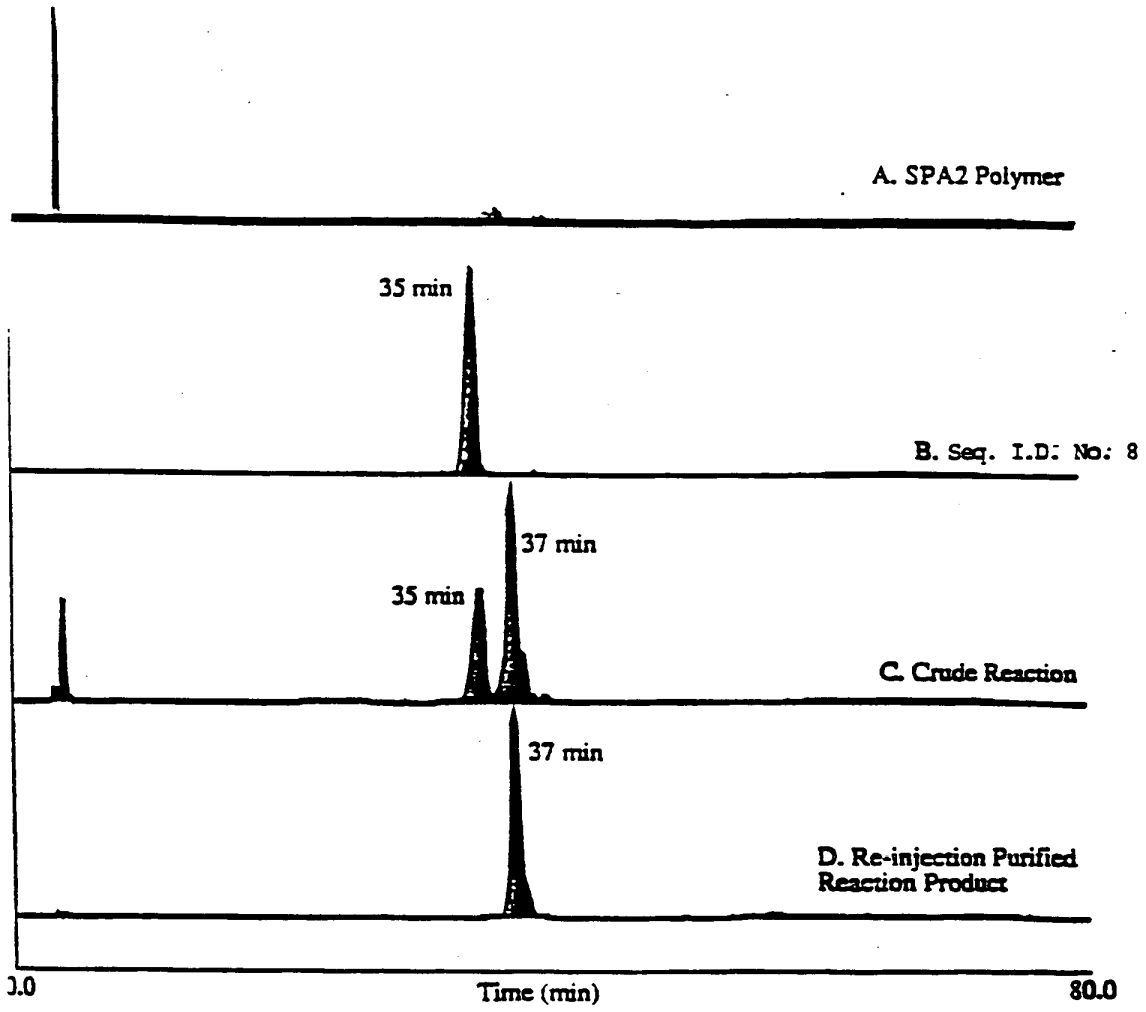


Figure 1

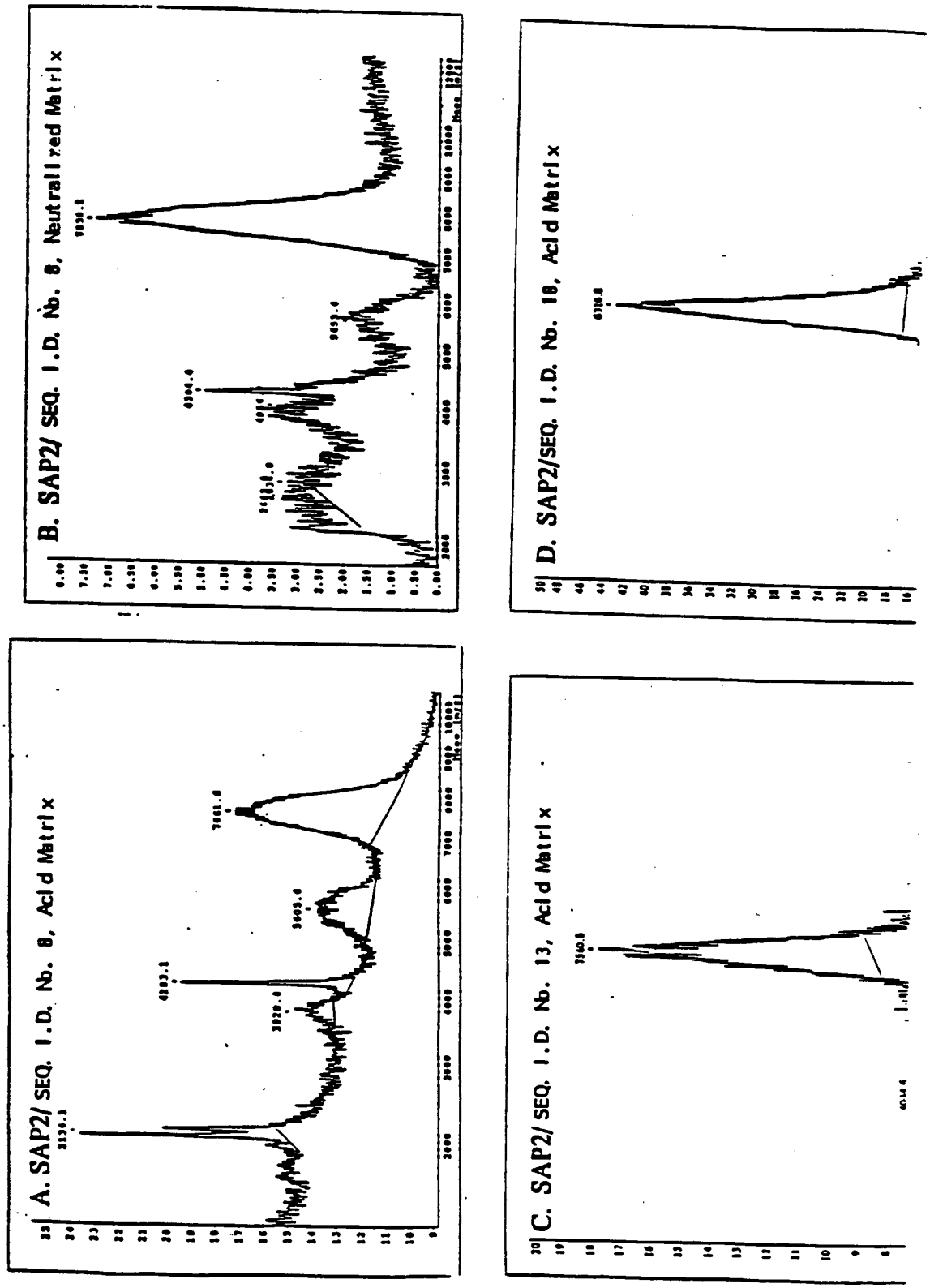

A. SAP2/ SEQ. I.D. No. 8, Acid Matrix
B. SAP2/ SEQ. I.D. No. 8, Neutralized Matrix
C. SAP2/ SEQ. I.D. No. 13, Acid Matrix
D. SAP2/SEQ. I.D. No. 18, Acid Matrix

Figure 3

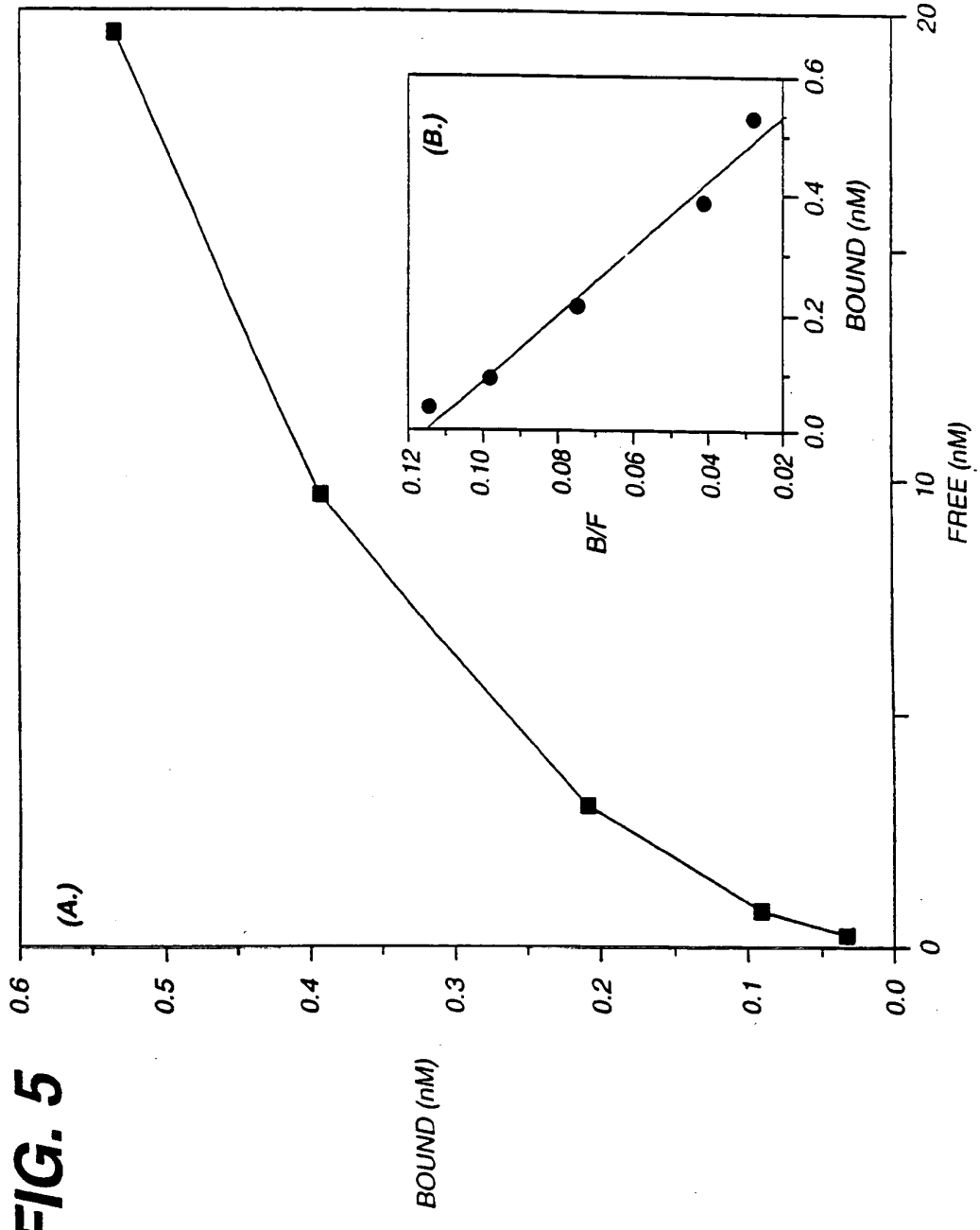


FIG. 5

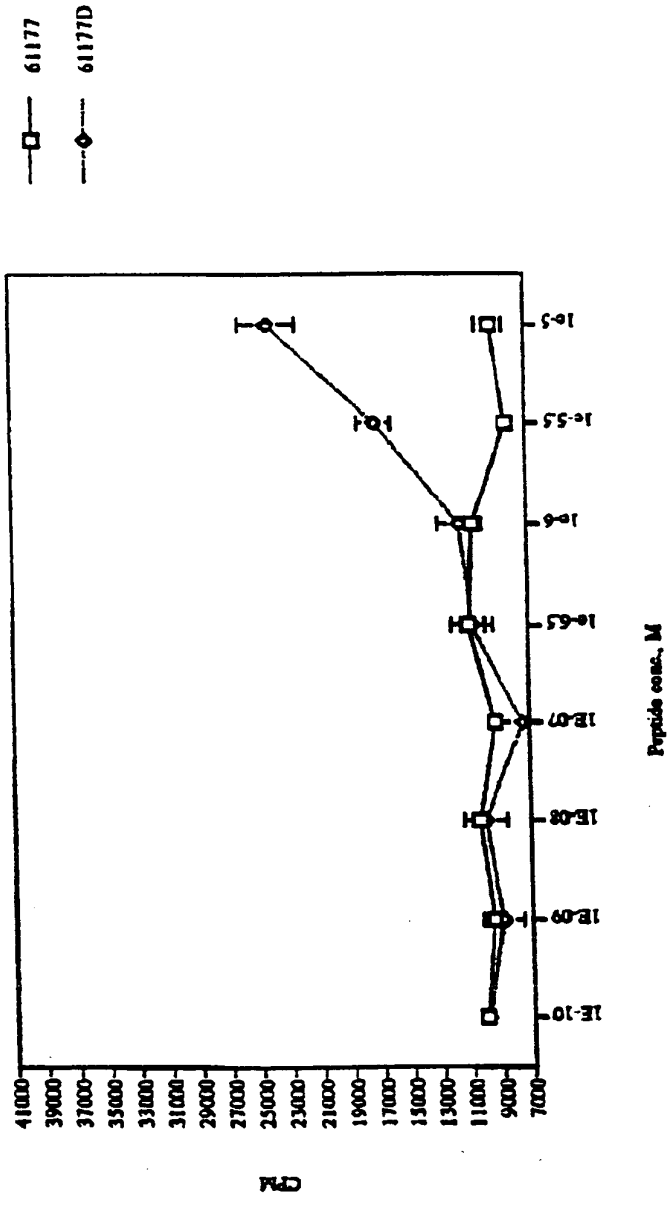


Figure 6
Panel D

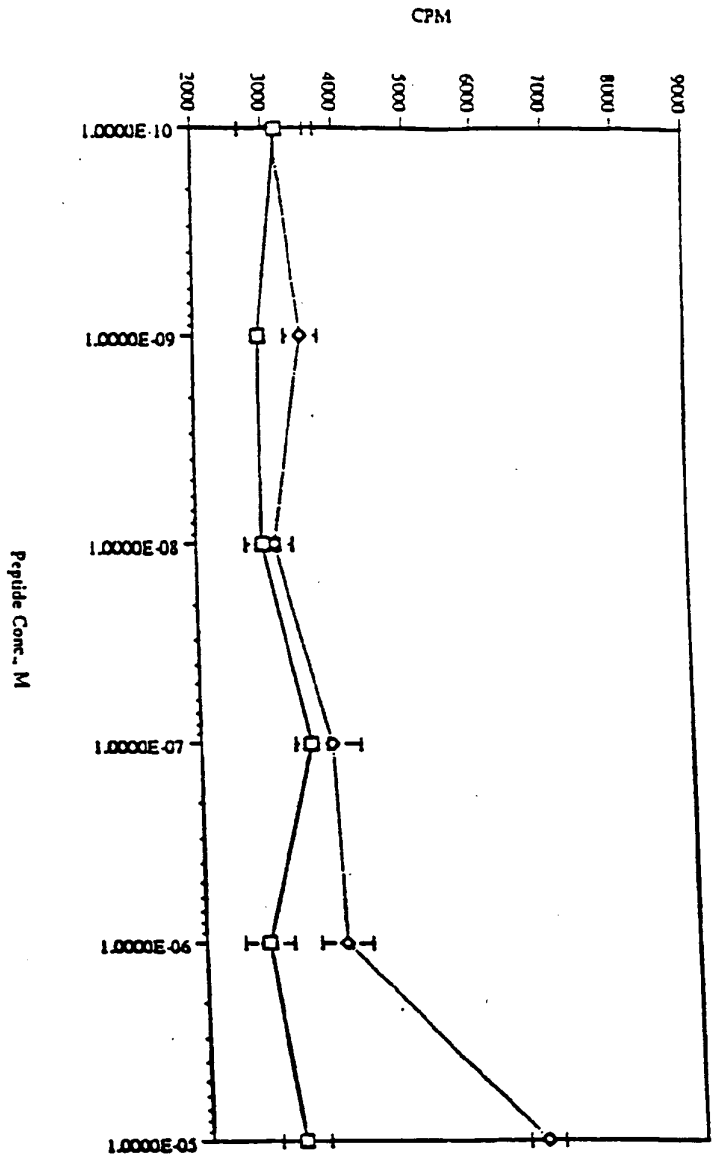


Figure 8

FIG. 9 (B)

GGVYKCRMGPLTWECRPTGG	SEQ ID NO: 61
GGDYNCRFGPLTWVCKPSGG	SEQ ID NO: 62
GGSYLCRFGPTTWLCSSAGG	SEQ ID NO: 63
GGSYLCRMGPTTWVCTRMGG	SEQ ID NO: 64
GGSYLCRFGPTTWLCTQRGG	SEQ ID NO: 65
GGWVTCRMGPITWVCGVHGG	SEQ ID NO: 66
GGQLLCGIGPITWVCRWVGG	SEQ ID NO: 67
GGKYSCFMGPTTWVCSPVGRGV	SEQ ID NO: 68
GGWVYCRIGPITWVCDTNGG	SEQ ID NO: 69
GGMYYCRMGPMTWVCKGAGG	SEQ ID NO: 70
GGTTQCWIGPITWVCRARGG	SEQ ID NO: 71
GGPYHCRMGPITWVCGPVGG	SEQ ID NO: 72
GGEYRCRMGPISWVCSPQGG	SEQ ID NO: 73
GGNYTCRFGPLTWECTPQGGGA	SEQ ID NO: 74
GGSWDCRIGPITWVCKWSGG	SEQ ID NO: 75
VGNYMCHFGPITWVCRPGGG	SEQ ID NO: 76
GGLYLCRMGPQTWMCQPGGG	SEQ ID NO: 77
GGDYVCRMGPMTWVCAPYGR	SEQ ID NO: 78
GGWYSCLMGPMTWVCKAHRG	SEQ ID NO: 79
GGKYYCWMGPMTWVCSPAGG	SEQ ID NO: 80
GGYVMCRIGPITWVCDIPGG	SEQ ID NO: 81
GSCLQCCIGPITWVCRHAGG	SEQ ID NO: 82
GGNYFCRMGPITWVCCPSVG	SEQ ID NO: 83
GGEYICRMGPLTWECKRTGG	SEQ ID NO: 84
GGLYACRMGPITWVCKYMAG	SEQ ID NO: 85
GGQYLCTFGPITWLCRGAGG	SEQ ID NO: 86
GGVYACRMGPITWVCSPLGG	SEQ ID NO: 87
GGYTTCRMGPITWVCSAHGG	SEQ ID NO: 88
GGTYKCWMGPMTWVCRPVGG	SEQ ID NO: 89
GGNYYCRFGPITFECHPTGG	SEQ ID NO: 90
GGEYLCRMGPMTWVCTPVGG	SEQ ID NO: 91
GGLYTCRMGPITWVCLPAGG	SEQ ID NO: 92
GGLYTCRMGPITWVCLPAGG	SEQ ID NO: 93

VERSION

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 7/06, 7/08, 14/00, 14/505, 14/52, 1/107, A61K 38/04, 38/16, 38/18

A3

(11) International Publication Number: WO 96/40772

(43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/US96/09469

(22) International Filing Date: 6 June 1996 (06.06.96)

(30) Priority Data:
08/484,135 7 June 1995 (07.06.95) US

(71) Applicant *(for all designated States except US)*: JOHNSON & JOHNSON [US/US]; One Johnson & Johnson Plaza, New Brunswick, NJ 08933-0001 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: JOHNSON, Dana, L. [US/US]; 1343 Lonely Cottage Road, Upper Black Eddy, PA 18972 (US). ZIVIN, Robert, A. [US/US]; 6 Glenbrook Court, Lawrenceville, NJ 08648 (US).

(74) Agents: CIAMPORCERO, Audley, A., Jr. et al.; Johnson & Johnson, One Johnson & Johnson Plaza, New Brunswick, NJ 08933-0001 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
31 July 1997 (31.07.97)

(54) Title: AGONIST PEPTIDE DIMERS

(57) Abstract

The present invention is directed to the dimerization of agonists and antagonists of cell surface receptors and particularly to peptide dimers which behave as cell surface receptor agonists in their dimeric form. Such receptors belong to the dimerization-mediated activation class often observed among receptors for growth and differentiation factors. The agonists of this class of receptors is understood to effect dimerization of the receptor and thus signal initiation. The present invention exemplifies dimers of erythropoietin (EPO) agonists and antagonists comprising a core amino acid sequence of $X_3X_4X_5GPX_6TWX_7X_8$ (SEQ ID NO: 1) wherein each amino acid is indicated by standard one letter abbreviation; X_3 can be C, A, α-amino-γ-bromobutyric acid or Hoc; X_4 can be R, H, L or W; X_5 can be M, F, or I; X_6 is idependently selected from any one of the 20 genetically coded L-amino acids or the stereoisomeric D-amino acids; X_7 can be D, E, I, L or V; and X_8 can be C, A, α-amino-γ-bromobutyric acid or Hoc, provided that either X_3 or X_8 is C or Hoc.

* (Referred to in PCT Gazette No. 07/1997, Section II)

BNSDOCID: <WO__9640772A3_I_>

INTERNATIONAL SEARCH REPORT

In: ional Application No
PCT/US 96/09469

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K7/06 C07K7/08 C07K14/00 C07K14/505 C07K14/52
C07K1/107 A61K38/04 A61K38/16 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 93 23550 A (GENENTECH INC ;GODOWSKI PAUL J (US)) 25 November 1993 see page 1 - page 34; claims 1-52 ---	15-19, 26-28 25
X Y	WO 95 11987 A (INCYTE PHARMA INC ;SCOTT RANDY W (US); BRAXTON SCOTT M (US)) 4 May 1995 see page 51 - page 61; claims 30-37; examples F,H,I ---	15-18 25
X	WO 90 08822 A (GENETICS INST ;WHITEHEAD INST (US)) 9 August 1990 see page 12, line 4 - page 12, line 27; claim 14 --- -/--	26,28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

4

Date of the actual completion of the international search	Date of mailing of the international search report
5 March 1997	17.06.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer GROENENDIJK, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 96/09469

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	SCIENCE, vol. 273, 26 July 1996, LANCASTER, PA US, pages 464-471, XP002021035 O.LIVNAH E.A.: "Functional mimicry of a protein hormone by a peptide agonist: the EPO receptor complex at 2.8 angström" see the whole document ---	1-30
T	SCIENCE, vol. 273, 26 July 1996, LANCASTER, PA US, pages 458-463, XP002021036 N.C.WRIGHTON E.A.: "Small peptides as potent mimetics of the protein hormone erythropoietin" see the whole document -----	1-30

4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Subject 1:claims 1-14,18,20-24,28-30(complete);15-17,25,26(all partially)
Compounds defined in the claims 1-6, their preparation and use and the methods defined in the claims 15-18,25,26 and 28, wherein the cell surface receptor is EPO-R.

Subject 2:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a GH (ant)agonist.

Subject 3:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a PDGF (ant)agonist.

Subject 4:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a EGF (ant)agonist.

Subject 5:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a G(M)-CSF (ant)agonist.

Subject 6:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a TPO (ant)agonist.

Subject 7:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a VEGF (ant)agonist.

Subject 8:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a FGF (ant)agonist.

Subject 9:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is an insulin (ant)agonist.

Subject 10:claims 15-17,19,25-27(all partially)
The methods defined in the claims 15-19 and 25-28, wherein the cell surface receptor (ant)agonist is a IL-3 (ant)agonist.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/09469

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9323550 A	25-11-93	US 5316921 A	31-05-94
		US 5328837 A	12-07-94
		EP 0642580 A	15-03-95
		EP 0642585 A	15-03-95
		JP 7508420 T	21-09-95
		JP 7508178 T	14-09-95
		US 5547856 A	20-08-96
		WO 9323541 A	25-11-93
		US 5580963 A	03-12-96
WO 9511987 A	04-05-95	AU 8076994 A	22-05-95
		EP 0730660 A	11-09-96
WO 9008822 A	09-08-90	US 5378808 A	03-01-95
		US 5278065 A	11-01-94
WO 9603438 A	08-02-96	AU 3149995 A	22-02-96
		CA 2195868 A	08-02-96
		EP 0773962 A	21-05-97
WO 9525746 A	28-09-95	US 5580853 A	03-12-96
		EP 0751959 A	08-01-97
US 4618598 A	21-10-86	NONE	
WO 9417099 A	04-08-94	US 5420243 A	30-05-95
		AU 6093294 A	15-08-94
		CA 2153789 A	04-08-94
		JP 8510443 T	05-11-96